The role of deubiquitinating enzymes in disease processes

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TABLE OF CONTENTS

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Tonje Folkvang

ABSTRACT

Ubiquitin (Ub) is a small protein which occurs in all eukaryotic cells (1). The biochemical function of Ub was first linked with protein turnover, but it has later been shown that Ub plays an important role in the control of many processes such as cell-cycle progression, transcriptional regulation and receptor down-regulation (2). A cascade of ubiquitin ligases conjugates monoubiquitin or, more commonly, ubiquitin chains to proteins. These molecules can then be removed by different kinds of deubiquitinating enzymes (DUBs).

The development of ubiquitin-derived thiol-reactive probes, which bind covalently to the catalytic cysteine of certain types of DUBs, has made it possible to analyze the composition of DUBs in different types of tissues and cells. The desired probe can be added to the tissue/cell lysate and allowed to react with the different DUBs before the modified enzymes are separated by SDS-Page and visualized by western blotting.

My supervisor and co-workers had been working with the HAUbVME probe before I joined them in the Intracellular Signaling Laboratory, Imperial College. They had discovered two DUBs (termed 50 kD and 60 kD), which where present in proliferating CD4+ T-cells but not in non-proliferating T-cells. They had a theory that one of these proteins corresponded to DUB-2 which is a T-cell specific DUB involved in proliferation and differentiation of lymphocytes (3). By using a probe-binding technique and western blotting for DUB-2 sequences we found that neither of these two enzymes were DUB-2. An experiment on human umbilical vein endothelial cells (HUVEC) showed that neither the 50 kD nor 60 kD DUBs of interest were found in endothelial cells. We concluded that it is likely that these DUBs are T-cell specific.

The existence of nuclear-specific DUBs has never been shown before. We ran a probe-binding reaction on nuclear extracts from cells synchronized in different stages of the cell cycle and found two DUBs in the nuclear lysates which were not present in the cytosolic control sample. We also discovered a DUB that was restricted to the G1 phase of the cell cycle. This DUB was also found in

the cytosolic lysate. Otherwise there did not seem to be any obvious differences in the levels and types of DUBs in the different stages of the eukaryotic cell cycle.

An investigation of the levels of active DUBs in inflammatory bowel disease (IBD) showed that FAM is dysregulated in intermediate colitis (IC). The levels of ubiquitinated proteins were not altered in IBD compared to healthy controls.

We also used the probe-binding technique to analyze tissues obtained from patients with ischemic heart disease (IHD), dilated cardiomyopathy (DCM) and transplant donors. We compared the levels of several ubiquitin proteases among these tissues by western blotting and found several interesting differences. HAUSP and USP14 were completely missing in the IHD samples, and the levels of USP15, UCH37 and UCH-L3 were clearly decreased. The level of UCH-L1 was normal. Some of the control samples also showed a decrease in the levels of DUBs, but we believe this was caused by ischemic conditions developed during transplantation or transportation. A western blot for Ub showed that the levels of ubiquitinated proteins were higher for the DCM samples then for the IHD samples and controls, which was consistent with what was found in the literature.

To recreate the decreased levels of active DUBs found in the IHD samples we grew HUVEC in an environment with only 1% oxygen. 96 hours of hypoxia was necessary to cause decreased levels of DUBs, though the decrease was not as profound as for the IHD samples. A western blot for Ub showed that more than 16 hours of hypoxia leads to a decreased level of ubiquitinated proteins in HUVEC. This result was unexpected as one would think that decreased levels of USP14 and UCH37, which are both involved in proteasome function, would lead to increased levels of ubiquitinated proteins.

The transcription factor NF-κB is tightly regulated by the ubiquitin proteasome system. It is also known that IL-1 induces the translocation of NF-κB from the cytoplasm to the nucleus (4). We decided to investigate whether changes in the ubiquitin-proteasome system seen in hypoxia correlate with perturbations in NF-κB translocation. We grew duplicate set of HUVEC on coverslips in an environment with only 1% oxygen. Each duplicate dish received IL-1 for the final

30 minutes of the incubation. Hypoxia did not inhibit the translocation of NF-κB in response to IL-1, however it turned out that hypoxia inhibits the constitutive translocation of NF-κB.

ABBREVIATIONS

ABS	Absorbance
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CD	Crohns' disease
cDNA	Complementary DNA
C-terminal	Carboxy terminal
DCM	Dilated cardiomyopathy
DUB	Deubiquitinating enzyme
Gad	Gracile axonal dystrophy
GAPDH	Glyceraldehydes-3-phosphate dehydrogenase
HA	Influenza hemagglutinin
HBSS	Hanks balanced salt solution
HRP	Horseradish peroxidase
HU	Hydroxyurea
HUVEC	Human umbilical vein endothelial cell
IBD	Inflammatory bowel disease
IC	Intermediate colitis
IL-1	Interleukin 1

IL-2	Interleukin 2
IHD	Ischemic heart disease
LB	Luria bertuni
SDS	Sodium dodecyl sulphate
TGF-ß	Transforming growth factor ß
Ub	Ubiquitin
Ubl	Ubiquitin-like protein
UBP	Ubiquitin-specific processing protease
UC	Ulcerative colitis
UCH	Ubiquitin C-terminal hydrolase
USP	Ubiquitin specific protease

1. INTRODUCTION

1.1 The ubiquitin system

1.1.1 Ubiquitin

Ubiquitin (Ub) is a small protein composed of 76 amino acids universally distributed among eukaryotes (1). The name ubiquitin was given to the protein because it is present in many tissues and organisms (2,5). Ub is highly conserved among eukaryotes. For example, only three amino acids differ between yeast and human Ub. This strong sequence conservation suggests that the vast majority of amino acids that make up Ub are essential to its function, as apparently any mutations that have occurred over evolutionary history have been removed by natural selection. Ub can exist either in a free form or as part of a complex with other proteins (1) (Figure 1.1.1).

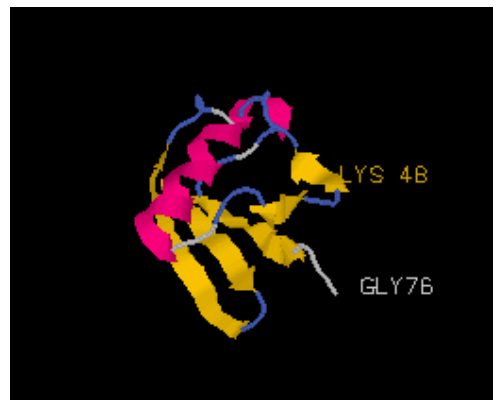

Figure 1.1.1: The Ub backbone showing lysine-48 to which further copies of Ub can be attached via an isopeptide bond with the C-terminus of another Ub. The C-terminal glycine-76 is also shown. Adapted from (6).

Ub was originally assumed to participate in the differentiation of lymphocytes. It was later found that Ub and ubiquitin-like proteins (Ubl) are conjugated post-translationally onto appropriate substrates and thereby regulating a wide variety of cellular processes (7). The biochemical function of ubiquitin was first linked with protein turnover. Ubiquitin-mediated degradation of regulatory

proteins is involved in many cell processes. It plays important roles in the control of processes such as cell-cycle progression, signal transduction, transcriptional regulation, receptor down-regulation and endocytosis. More recently the ubiquitin-system has also been implicated in the immune response, development and apoptosis. Dysfunction in several ubiquitin-mediated processes may have a causal role in many human diseases, including a variety of cancer (2).

1.1.2 Ubiquitination

Ub is conjugated to proteins through a covalent isopeptide bond between the glycine at the C-terminal end of Ub (see Figure 1.1.1) and a lysine side chain or, less commonly, the N-terminus of the substrate protein (7) (Figure 1.1.2).

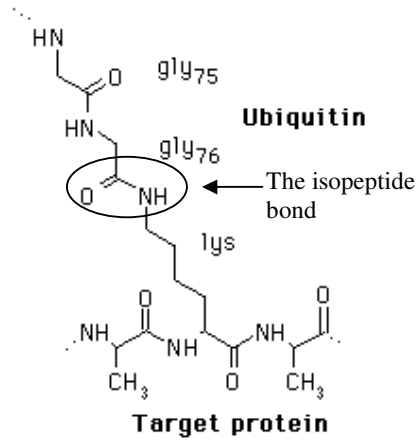

Figure 1.1.2: A representation of the isopeptide bond between Ub and a target protein. Ub glycine-75-glycine-76 coming down from the top left, N to C. Target protein along the bottom, left to right, N to C, shown with alanines flanking the acceptor lysine. Adapted from (8).

Ubiquitin conjugation is ATP-dependent and is achieved by the sequential actions of members of three enzyme families (7). E1 enzymes, known as ubiquitin-activating enzymes, modify Ub so that it is in a reactive state, which makes it likely that the C-terminal glycine on Ub will react with the lysine side-chains on the substrate protein. Most organisms contain one or two such enzymes. The activated Ub is then passed on to one of several dozen ubiquitin-conjugating enzymes, or E2

enzymes. These activated E2 enzymes are bound by one of several hundred E3 enzymes, known as ubiquitin-ligases (9). Some E3 enzymes are associated with large multisubunit complexes. Specific E3 enzymes appear to be responsible mainly for the selectivity of ubiquitin-protein ligation. They do so by binding specific protein substrates that contain specific recognition signals. In some cases, binding of the substrate protein to an E3 is indirect, via an adaptor protein. Different types of E3 enzymes may carry out the transfer of Ub to the substrate protein by at least two different mechanisms. In some cases, E3 accepts the activated Ub from an E2 prior to transfer to protein, while in others the Ub is transferred directly from E2 to a protein (5). Additional ubiquitin molecules can be attached in similar fashion to lysine residues of the first Ub, and multiple rounds of conjugation result in the formation of branched polymeric ubiquitin chains. The final result is ubiquitinated proteins that can participate in DNA repair, gene expression, endocytosis, be degraded by the proteasome etc. (10) (Figure 1.1.3).

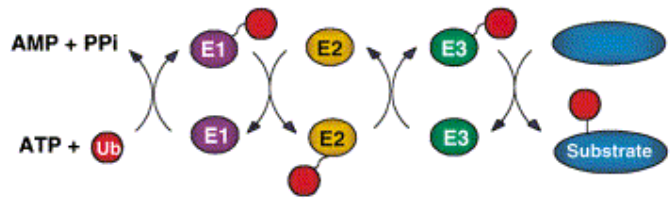

Figure 1.1.3: A simplified schematic description of the ubiquitination cascade. Adapted from (10).

Although single Ub molecules can be conjugated to proteins, more commonly ubiquitin-chains are being be attached. Monoubiquitination is known to regulate several divergent cellular activities, including endocytosis of surface receptors, activation of caspases and inhibition of some protein-protein interactions. Different types of polyubiquitin chains can be formed depending on the lysine residue used to form the bonds between the different ubiquitin molecules (Figure 1.1.4).

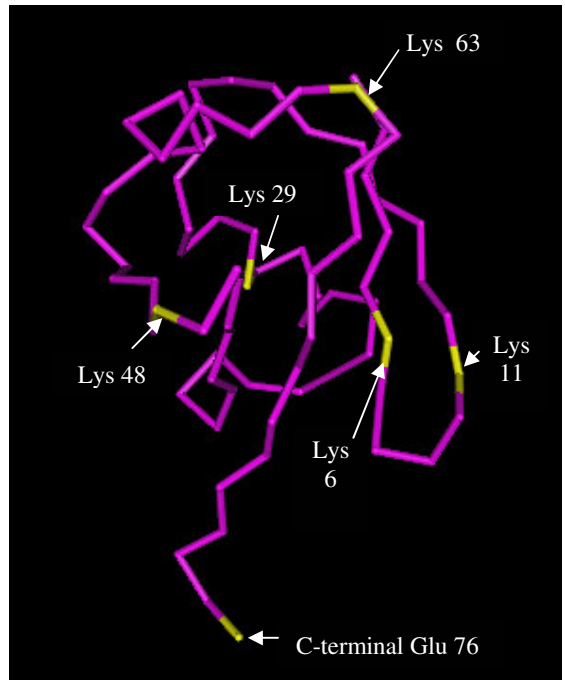

Figure 1.1.4: The structure of ubiquitin showing the C-terminal glycine and the polyubiquitination sites (lysines). *Glu*, glycine. *Lys*, lysine. Obtained from (11).

Attachment of lysine-48-linked polyubiquitin chains targets proteins for degradation by the proteasome, while 'nonclassical' chains linked through lysine-6 or lysine-63 participate in DNA repair processes. Lysine-63-linked polyubiquitin also regulates the activity of several pro-inflammatory signaling molecules. Ubiquitin chains can also be synthesized with linkages between the C-terminus of Ub and lysine-11 and lysine-29 on the adjacent Ub. The precise role of these linkages is yet unknown (9) (Figure 1.1.5).

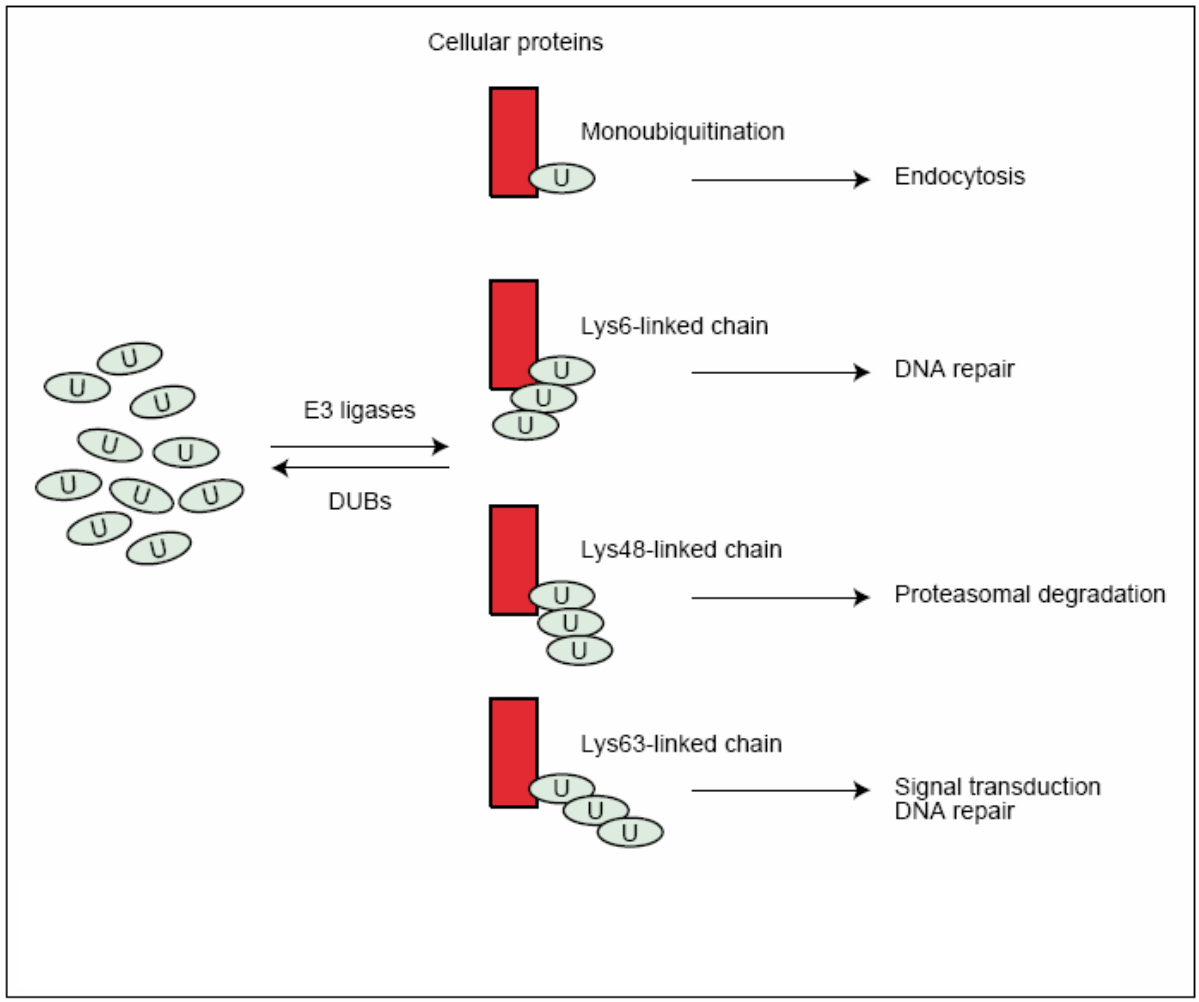

Figure 1.1.5: Ub can be attached to lysine residues of cellular proteins either as monoubiquitin or as different forms of polyubiquitin. The different forms of Ub all confer distinct effects on the proteins to which they are attached. *U*, Ubiquitin. Adapted from (9).

Ubiquitination of proteins often causes them to interact with other cellular proteins that contain ubiquitin-binding domains. There are a large number of such proteins, which reflects the importance of ubiquitination in regulating multiple physiological processes. Ubiquitin binding molecules regulate numerous activities, including proteasomal degradation, endocytosis, DNA repair, mRNA splicing, intracellular trafficking and intracellular signaling. It is possible that particular ubiquitin-binding domains might bind preferentially to monoubiquitin or to specific forms of polyubiquitin. This might contribute to the different physiological effects of distinct forms of ubiquitination (9).

Identification of a protein for ubiquitination may in many cases involve a genetically encoded ubiquitination signal and/or a prior modification such as phosphorylation or binding to an adapter protein, or damage to the protein due to fragmentation, oxidation or aging (1).

1.1.3 Targeting of proteins for degradation by the proteasome

Protein degradation is essential to the cell to supply amino acids for fresh protein synthesis, to remove excess enzymes, transcription factors or other cellular proteins that are no longer needed and for other fundamental physiological processes. Ub is involved in two of the intracellular organelles in which damaged or unneeded proteins are broken down: lysosomes and proteasomes. While lysosomes primarily deal with extracellular proteins, proteasomes deal mainly with cytoplasmic proteins, that is, proteins localized within the cell (9).

The proteasome is a highly selective, large multisubunit protease complex localized in the cytoplasm and nucleus (12). It can be compared to a recycling bin were old/damaged proteins are delivered at the top while peptides and amino acids, which can be used for the synthesis of new proteins, come out at the bottom.

The proteasome is composed of two complex components, the cylindrical 20S core particle and a 19S cap particle which docks onto both ends of the barrel-shaped 20S, to yield the 26S proteasome. The 26S proteasome is viewed as the biologically active unit. The active sites of the proteasome are protected from the cellular environment in the interior of the 20S subunit. Proteins are recognized by the regulatory 19S complexes trough the binding of the lysine-48-linked polyubiquitin chain attached to the proteins. The 19S subunit unfolds the protein substrates and assists in their translocation through a narrow gate in the 20S core particle where degradation takes place. The protein substrates are degraded processively until only small peptides or amino acid residues remain. The 19S complex also contains deubiquitinating enzymes that remove Ub from the substrate protein. The 26S proteasome complex requires ATP hydrolysis for its action (12) (Figure 1.1.6).

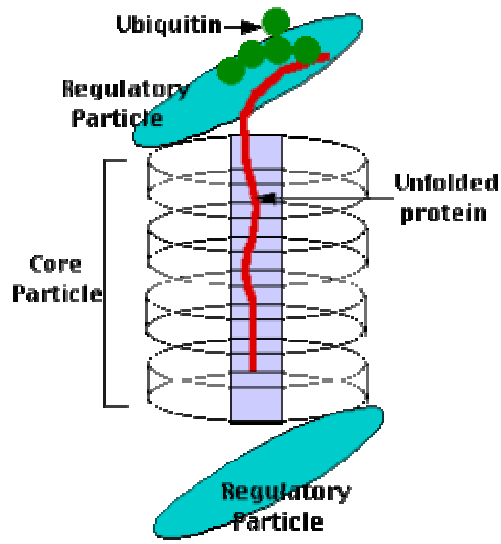

Figure 1.1.6: A schematic representation of the 26S proteasome. The regulatory particle corresponds to the 19S subunit and the core particle corresponds to the 20S subunit. Adapted from (13).

The action of the 26S proteasome presumably generates several types of products, such as free peptides, short peptides still linked to Ub via their lysine residues and polyubiquitin chains. These products can then be further processed by different enzymes to yield useful building blocks or biological active products (5). For example, the ubiquitin-proteasome pathway serves as the major source of peptides during antigen presentation by major histocompatibility complex (MHC) class 1 molecules. Besides making useful degeneration products it is now also widely recognized that the proteasome also functions to destroy specific cellular proteins in response to extracellular or intracellular signals. For example, the ubiquitin-proteasome complex is thought to conduct rapid changes in the composition of cytosolic or nuclear protein pools during mitosis, differentiation, apoptosis, responses to growth factors or cytokines and many other physiological activities (9) (Figure 1.1.7).

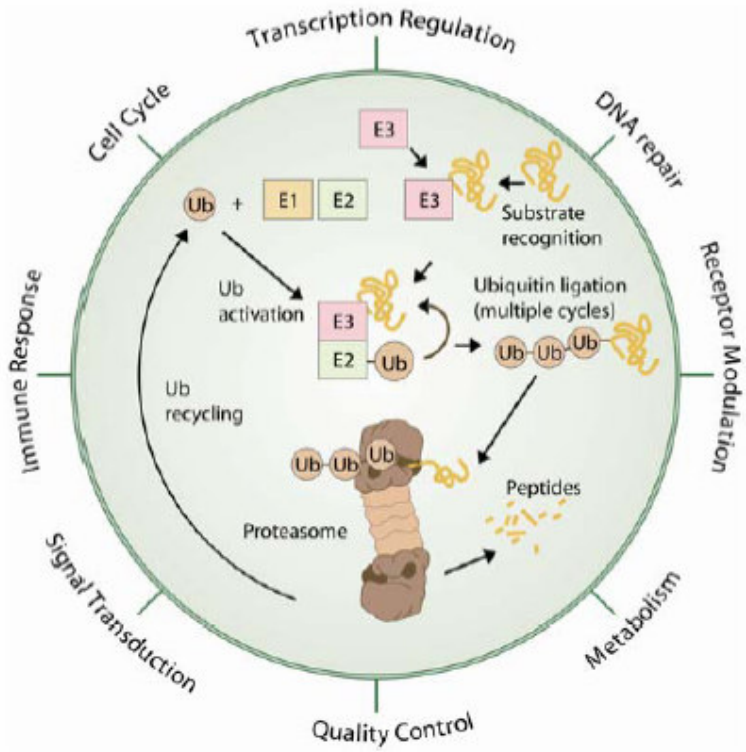

Figure 1.1.7: Ubiquitin-mediated proteolysis and its many biological functions. A summary of the ubiquitin system. Adapted from (12).

1.1.4 Deubiquitination

The ubiquitin system is also regulated by deubiquitinating enzymes (DUBs), which remove Ub or Ubl from attached proteins by cleavage of the isopeptide bond (Figure 1.1.8).

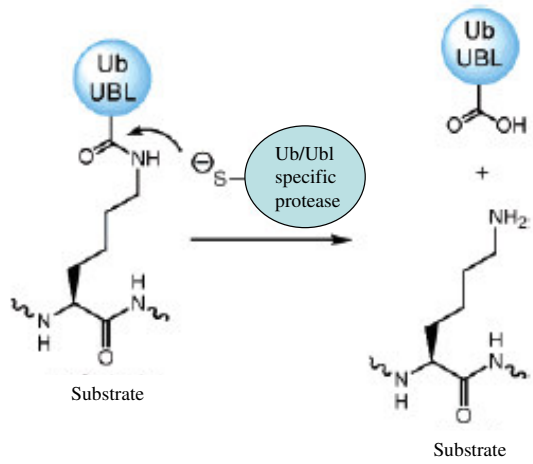

Figure 1.1.8: Protein substrates conjugated with Ub or Ubl are subjected to proteolysis by a set of specific proteases. *UBL*, ubiquitin-like proteins (Ubl) Adapted from (7).

DUBs have many roles, for example the precursors of monomeric ubiquitin are expressed as either fusions with certain ribosomal proteins or head-to-tail-linked ubiquitin multimers that also have an additional amino acid following the last ubiquitin monomer. Proper processing of these precursors is essential for the generation of conjugation-competent Ub monomers. Second, the C-terminus of Ub must be regenerated after degradation of the attached protein and any adventitious reactions of ubiquitin thiol esters with cellular amines and thiols must be reversed by processing at the C-terminus. Third, DUBs could act as 'proof-reading' enzymes to reverse ubiquitination of the 'wrong' protein, or even the 'right' protein at an inappropriate time or place. Finally, the polyubiquitin chain must be disassembled after use to replenish the ubiquitin pool and prevent accumulation of free chains that could act as competitive inhibitors of the binding of ubiquitinated proteins to the proteasome (1) (Figure 1.1.9).

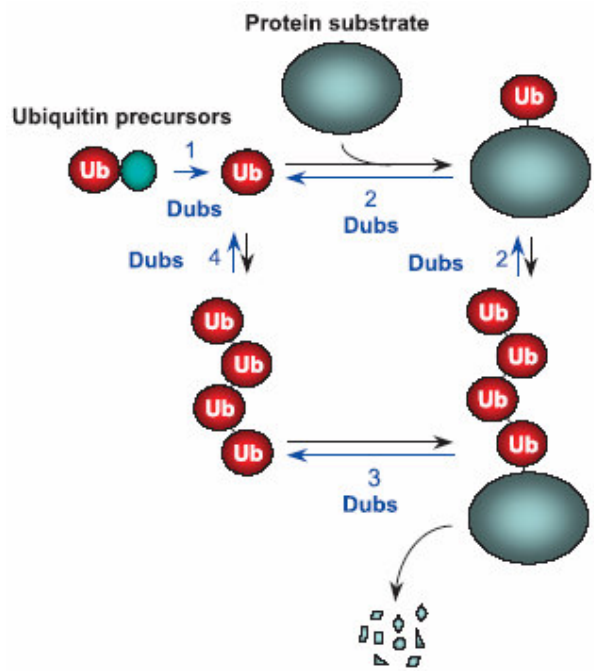

Figure 1.1.9: Some of the functions of deubiquitinating enzymes in the ubiquitin pathway. (1) Processing of ubiquitin precursors. (2) Editing or rescue of ubiquitin conjugates. (3) Recycling of Ub or ubiquitin oligomers from ubiquitin-protein conjugates targeted for degradation. (4) Disassembly of unanchored ubiquitin oligomers. Adapted from (14).

The DUBs are a large group of proteins divided into at least five distinct subfamilies based on their sequence similarities and likely mechanisms of action. Four of these five subfamilies consist of specialized cysteine proteases, which are characterized by the presence of catalytically active cysteine residues. The fifth group, on the other hand, is a novel type of zinc-dependent metalloprotease (14).

The largest and most diverse of these sub-families is the ubiquitin specific processing proteases (UBPs). The UBPs are cysteine proteases and they contain two short but well-conserved motifs, named the Cys and His boxes, which include the active site (14). The UBPs are thought to be responsible for removing Ub from larger proteins and disassembling the polyubiquitin chains. UBPs regulate signal transduction, growth and development. The size of these proteins varies and some of them have a C-terminal extension which is thought to contribute to substrate specificity and/or localization (1).

The second subfamily of ubiquitin-specific cysteine proteases is made up of the ubiquitin C-terminal hydrolases (UCHs). They are generally small proteins and as the UBPs they contain a Cys and His box (14). The UCHs are thought to be involved in processing ubiquitin-precursor proteins and are active on Ub extended by small peptides or larger substrates with a flexible peptide linking the C-terminal domain. They are, however, poor at cleaving ubiquitin-protein conjugates. They have important roles in development and neural function (7). Mutations in this family of enzymes appear to be associated with disease. For example, a mutation in one type of human UCH may be involved in some cases of Parkinson's disease, and mutation in another UCH may be associated with some lung cancers (1).

The substrate specificities of UBPs and UCHs overlap. Both can associate with the 26S proteasome and are involved in the regulation of ubiquitin-dependent proteolysis (15).

The remaining three known subfamilies of DUBs have been discovered only recently, and hence less is known about these families and their activities. The ovarian tumor-related proteases (OTUs) are a novel family of cysteine proteases (14). These proteins display structural similarity in a presumed catalytic core domain containing conserved Cys, His and Asp residues thought to comprise the catalytic site. The OTU protease family includes members in which the OTU-related motif is actually part of a UBP family protein. One member of the OTU family has been shown to hydrolyze linear polyubiquitin translation products and isopeptide-linked polyubiquitin chains. It has also been shown to be active against ubiquitin-protein conjugates. Another OTU member has been shown to be active against both lysine-48- and lysine-63-linked ubiquitin oligomers (14). Future studies will tell us more about this family and its actions.

The last family of ubiquitin-specific cysteine proteases has only one member to date, Ataxin-3. Ataxin-3 is characterized by a domain called the Josephine domain, which includes segments that show weak similarity to the His and Cys boxes of UBPs and UCHs. Ataxin-3 disassembles ubiquitin-lysozyme conjugates, cleaves ubiquitin-7-amido-4-methylcoumarin and binds to the DUB inhibitor ubiquitin aldehyde (14).

The fifth, and last, subfamily of DUBs is represented by a subunit of the proteasome, Rpn11/POH1, which has features of a metalloprotease specific for protein-linked Ub (14). The active site in this family is called the JAMM motif and it includes two absolutely conserved His residues and an Asp residue that together coordinate a zinc ion important for proteolytic activity. These residues are essential for the function of the Rpn11 subunit when integrated into the proteasome. Another conserved residue, glutamate-48, is thought to serve as a general acid-base catalyst. Very recently, another protein with the JAMM motif, AMSH, was found to have deubiquitinating activity as well (14).

DUBs vary greatly in length and structural complexity, but all the cysteine proteases contain two conserved domains that make up the active site. The Cys domain contains a cysteine residue that serves as the active enzymatic nucleophile, while the His domain contains a histidine residue that contributes to the enzyme's active site (16).

The human genome encodes 60-70 predicted members of the DUB family. The large number of DUBs suggests that these enzymes may exhibit selectivity for the type of ubiquitin linkage hydrolyzed or the protein substrates acted on and may thereby regulate specific cellular processes. Indeed, specific substrates have been identified for some DUBs and it is clear that some DUBs exert distinct growth regulatory activities by acting as oncoproteins or tumor suppressor proteins (17).

Ubiquitin aldehyde is a specific inhibitor of many DUBs. Alkylating reagents, such as *N*-ethyl-maleimide and iodoacetamide, also inhibit many deubiquitinating activities, presumably through alkylation of the active site cysteine (18).

Thus it is becoming clear that a number of proteins regulating cellular mechanisms for homeostasis in all eukaryotes are controlled not only by phosphorylation and dephosphorylation but also by ubiquitination and deubiquitination. Like protein phosphorylation and dephosphorylation regulated by kinases and phosphatases, respectively, protein ubiquitination and deubiquitination are very dynamic and are regulated by ubiquitin conjugating enzymes and DUBs (Figure 1.1.10).

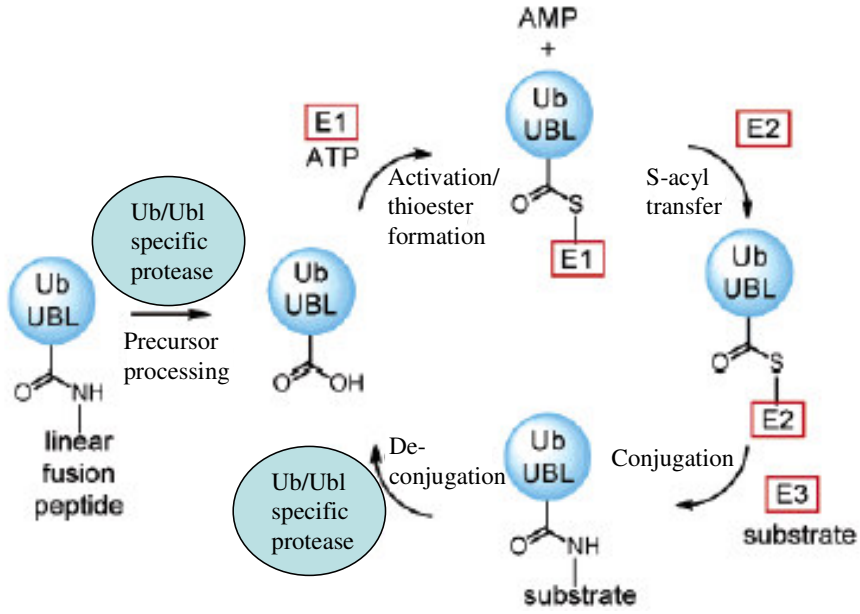

Figure 1.1.10: A summary of the ubiquitin pathway showing both ubiquitination and deubiquitination. Ub is synthesized as fusion proteins requiring the action of specific proteases to generate the mature ubiquitin molecules. Adapted from (7).

1.2 Ubiquitin-derived thiol-reactive probes

1.2.1 Ubiquitin-derived thiol-reactive probes

More than 60 DUBs have been predicted from sequence similarity searches in the human genome, but the biological role of most of these enzymes remains unknown. Elucidation of the function of individual DUBs is complicated by a considerable overlap in substrate specificity (19). Studies aimed at a functional characterization of putative DUBs and analysis of their expression and activity under different physiological and pathological conditions have been boosted by the development of influenza hemagglutinin (HA)-tagged, ubiquitin-derived, active-site-directed probes that allow covalent modification of the active enzymes, followed by their isolation and identification (17).

Seven different probes have been made, all composed of three elements: A full-length sequence of Ub which confers specificity for the deubiquitinating enzyme family, a thiol-reactive group that

allows covalent mechanism-based trapping of the active site cysteine of the cysteine proteases and a HA tag that enables the probe/protease complexes to be detected using HA antibodies (Figure 1.2.1).

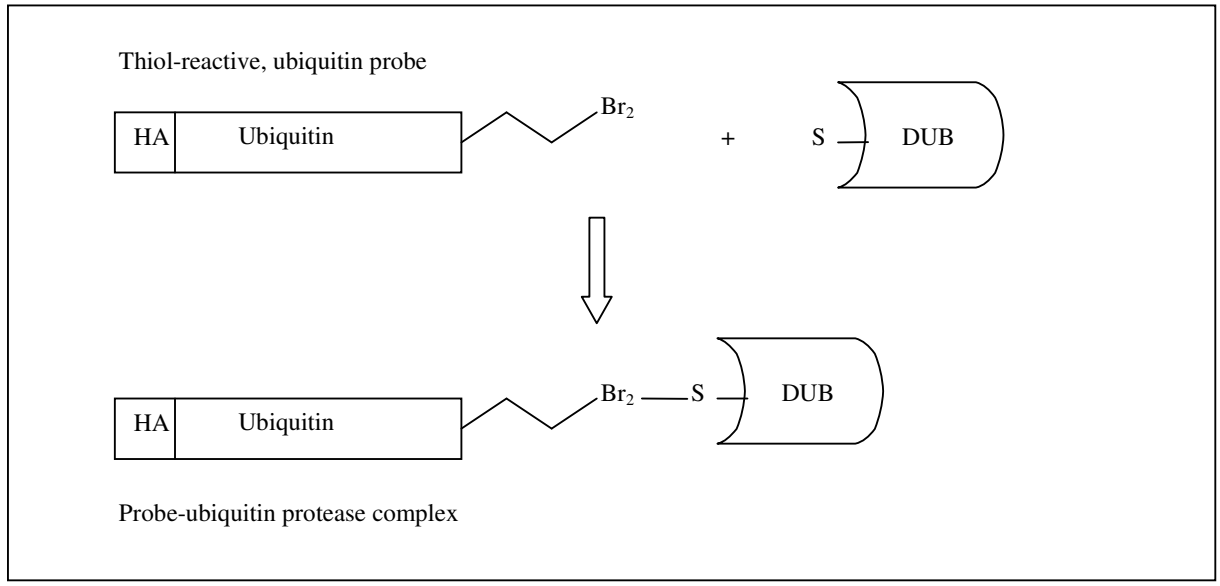

Figure 1.2.1: Schematic representation of one of the probes binding to a DUB. Figure provided by Paul Evans.

The theory behind the composition of these probes is that most DUBs can bind an ubiquitin monomer, even if the monomer is not the preferred substrate in vivo. In addition, all DUBs, except the subfamily containing the JAMM motif, are cysteine proteases that cleave the ubiquitin after the C-terminal glycine-glycine motif. Studies have indicated that the enzymes interact with large areas of Ub in addition to recognizing the cleavage site and therefore the entire sequence of Ub was chosen as a specificity element for the development of the probes (7). It has been shown that the presence of an HA tag does not adversely affect interaction with enzymes that utilize Ub (19).

The first probe synthesized (HAUbVS) had a vinylmethyl sulfone as the thiol-reactive group. Vinyl sulfones are mechanism-based cysteine protease inhibitors that form stable covalent complexes with target enzymes. This probe was designed so that the electrophilic carbon of the vinyl sulfone was positioned in the same place as the ubiquitin-terminal carbonyl moiety in the natural substrate, thus placing it in the optimal position for nucleophilic attack by the active site cysteine of a DUB. An active site cysteine residue was added onto the vinyl sulfone and this

resulted in the formation of a covalent and robust thioether adduct. It has been demonstrated that this probe covalently target a set of DUBs, but not all of them (7).

Because of the need to target more DUBs than what was possible with the HAUbVS probe, six other probes were synthesized. These had six different thiol-reactive groups to target a wide range of DUBs. The C-terminal traps in the seven different probes include four Michael acceptor-derived probes: vinylmethyl sulfone (HAUbVS), vinyl methyl ester (HAUbVME), vinyl phenyl sulfone (HAUbVSPh) and viny cyanide (HAUbVCN), and three alkylhalide-containing inhibitors: chloroethyl (HAUbCl), bromoethyl ($HAUbBr_2$) and bromopropyl ($HAUbBr_3$) (19). All these different probes have different reactivity toward DUBs, and the alkylhalide-containing probes modify a distinct set of polypeptides when compared to the Michael acceptors (7). HAUbVME exhibit the broadest reactivity, while $HAUbBr_2$ modifies a more restricted number of DUBs. Most polypeptides modified by HAUb-derived probes contain known sequence motifs characteristic of the UBP or UCH enzymes families. All proteins covalently modified by HAUb-derived probes are hydrolases. The probes do not target Ubiquitin activating- and conjugating enzymes. The fact that different electrophiles at the C-terminal end of Ub vary considerably in their ability to react with individual DUBs indicate that the active site of DUBs belonging to the same family are not equivalent and that it in the future may be possible to design more selective probes (19).

The probes were generated by using a chemical ligation method where the different Michael acceptors or alkylhalide derivatives were introduced at the C-terminus of Ub (7) (Figure 1.2.2).

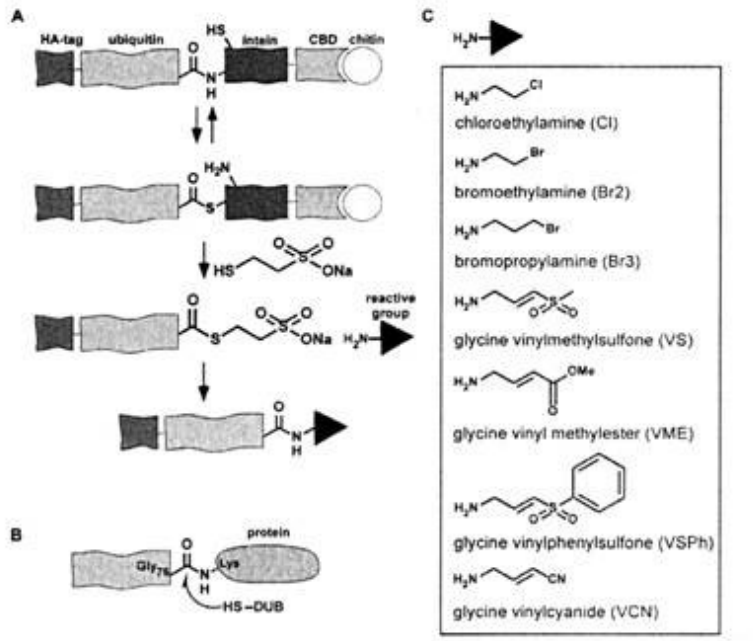

Figure 1.2.2: Synthesis of HAUb-derived probes. (A) The intein-based chemical ligation method. (B) Site of attack of a hydrolase on the peptide bond at the C terminus of Ub. (C) Structures of the C-terminal thiol-reactive groups used. Adapted from (19).

Modification by a HAUb-derived probe increases the size of a DUB by approximately 10 kD and the resulting branched polypeptide may migrate at a larger apparent molecular weight (7).

These HA epitope tagged probes make it possible to analyze the composition of DUBs in different types of tissues and cells. The desired probe can be added to the tissue/cell lysate and allowed to react with the different DUBs before the modified enzymes are separated by SDS-Page and visualized by western blotting, Coomassie blue staining or silver staining. In addition, probe-protease complexes may also be purified by immunoprecipitation using anti-HA antibodies (HA epitope is incorporated into the N-terminus of the probe). Following gel electrophoresis, probe-protease complexes may be stained and then excised and analyzed by tandem mass spectrometry to identify the different DUBs. Activity profiles of entire enzyme families active in crude extracts can be obtained with these probes in a single experiment. In addition, these probes can be used to retrieve and identify new members of the deubiquitinating enzyme families (7).

1.3 The function of deubiquitinating enzymes (DUBs)

1.3.1 DUB-2

DUB-2 is an UBP containing 545 aminoacids with an observed molecular weight of approximately 62 kD (16,20,21). DUB-2 appears to be specifically regulated by interleukin-2 (IL-2) and its expression is restricted to T-cells. Binding of IL-2 to its receptor results in activation of the JAK/STAT pathway, and this leads to expression of different genes, including the one for DUB-2. Many of these gene products are involved in proliferation and differentiation of lymphocytes while others act in a feedback loop to modulate the signaling. DUB-2 can enhance signaling specifically in response to IL-2 and can contribute to constitutive activation of the JAK/STAT pathway and enhanced lymphocyte survival. It has also been shown that DUB-2 can suppress apoptosis in lymphocytes following withdraw of growth factor. Data suggests that DUB-2 may play an important role in controlling T-lymphocyte survival and perhaps contribute to T-cell transformation (3).

1.3.2 HAUSP

HAUSP is also known as USP7. The size of this UBP is approximately 128 kD (19). HAUSP interacts with the tumor suppressor protein p53. Activation of this tumor suppressor protein results in cell cycle arrest and other alterations that eventually lead to programmed cell death. A defect in the function of p53, which allows pre-cancerous cells to survive and proliferate, eventually leading to a tumor, is a feature of many cancers (14). Cellular levels of p53 are normally maintained at a low steady state, but cellular stress results in rapid accumulation of the protein suggesting that p53 regulation is controlled primarily at the post-transcriptional level. Polyubiquitination and degradation by the proteasome is the principal mechanism underlying this post-transcriptional control. It was first thought that HAUSP could deubiquitinate p53 to avoid its degradation. Later studies raveled that a lack of HAUSP leads to increased, rather then decreased, levels of p53 due to decreased ubiquitination of p53. It is now believed that MDM2, a negative regulator of p53 levels, rather then p53 is the substrate for HAUSP. MDM2 possesses an E3 ligase activity and is believed

to ubiquitinate p53. Hence, HAUSPs effect on p53 may be secondary to MDM2, but it has been established that HAUSP is required for proper p53 regulation (22).

1.3.3 USP14

USP14 is a mammalian homolog of yeast Ubp6p, and it is also known as Ubp6. The size of this UBP is approximately 56 kD (15,23). USP14 is found in the 19S subunit of the proteasome and its deubiquitinating activity is strongly stimulated by proteasome binding. USP14 directly associates with the 19S regulatory particle via an ubiquitin-like domain at its N-terminus. Data suggests that USP14s participation in proteasome function is in an ancillary or partly redundant fashion (14). The precise physiological function of USP14 remains unclear. USP14 possess a type II ubiquitin-like domain at the N-terminus. This domain may be necessary for targeting USP14 to its interacting partner or substrate. Other proteins containing a type II ubiquitin-like domain require this for interaction with the 26S proteasome. The role of this domain in USP14-proteasome association is currently under investigation. Preliminary data suggest that this domain does not target USP14 for degradation by the proteasome (15).

Future experiments will reveal the truth about USP14s role in proteasome functional, but a functional coupling between the activities of the proteasome and proteasome-bound USP14 has been proposed. Preliminary data indicates that only the proteasome-bound form of USP14 can be labeled with the HAUbVS probe, suggesting that the affinity of proteasome-bound USP14 for Ub may be different from that of the free protein. If this is correct, USP14 is the first example of a DUB whose substrate specificity and activity are regulated by association with a binding partner (15).

1.3.4 USP15

USP15 is a UBP with an observed molecular mass of approximately 103 kD (17,19). To date there is not much known about the function of this protein. The sequence of USP15 is closely related to UNP, which is the human homolog of the mouse Unp proto-oncogen. The product of this mouse proto-oncogen has been shown to have DUB activity. UNP is able to cleave ubiquitin-proline bonds efficiently. It has been shown that USP15 is the only UBP that also has this ability.

The biological significance of this activity is at present unknown. UNP has been observed in primary tumor tissue from small cell tumors and adenocarcinomas of the lung, suggesting a possible causative role for UNP in these cancers. Notably, another study did not observe this elevated expression but rather found a slight reduction in UNP protein levels. Despite these different findings a role for UNP in regulating cell growth is apparent. UNPs have consensus retinoblastoma protein interaction motifs within their sequences. USP15 also contains these motifs, and it has been proposed that USP15 may also have a similar growth regulatory role as UNP (24).

1.3.5 UCH37

As the name suggests, UCH37 is an UCH enzyme (12). The observed size of this DUB is approximately 38 kD (17). UCH37 is a mammalian homolog of the yeast Uch2. In conformity with USP14, UCH37 is involved in proteasome function, but as opposed to USP14, UCH37 does not have a type II ubiquitin-like domain at the N-terminus. UCH37 is most likely an integral subunit of the 26S proteasome. It has been claimed that UCH37 is responsible for the majority of the deubiquitination activity to the proteasome. However, when the genes encoding the UCH37 analog in fission yeast are disrupted, the cells are viable without showing obvious signs of impaired ubiquitin-dependent proteolysis, indicating that other DUBs may remedy for the redundancy of this enzyme (25).

1.3.6 UCH-L1

UCH-L1 is an UCH of approximately 25 kD that is selectively expressed in the testis/ovary and neuronal cells. It is extremely abundant in the brain but its exact roles there are uncertain. UCH-L1 has a relatively weak deubiquitinating activity, but in addition it exhibits dimerization-dependent ubiquitin ligase activity. Studies have revealed that gracile axonal dystrophy (*gad)* mice, which lack UCH-L1 expression, show reduced retinal cell apoptosis in response to ischemia, suggesting that UCH-L1 may promote apoptosis. Indeed, it has been suggested that UCH-L1 has anti-proliferative activity in tumor cells and that its expression is induced in response to tumor growth. Another study on *gad* mice demonstrated that UCH-L1, together with UCH-L3, is involved in the regulation of the cellular levels of anti-apoptotic, prosurvival and apoptotic proteins during testicular germ cell

apoptosis. It has also been shown that UCH-L1 associates with monoubiquitin and prolongs ubiquitin half-life in neurons (15,19,26).

1.3.7 UCH-L3

UCH-L3 is an UCH of approximately 26 kD (19). It shares significant structural similarity with UCH-L1, but as oppose to UCH-L1 it is distributed ubiquitously throughout the body. Both UCH-L1 and UCH-L3 are strongly but reciprocally expressed in the mouse testis during spermatogenesis, and it has been shown that they regulate the levels of different proteins during testicular germ cell apoptosis. The activity of UCH-L3 is more than 200-fold higher than UCH-L1 when a fluorogenic ubiquitin substrate is used, but UCH-L3 has little or no ligase activity. UCH-L3 has activity against Nedd8, which is an ubiquitin-like protein. UCH-L3 binds Nedd8 and subsequently processes its C-terminus. Covalent conjugation of proteins by Nedd8 is an important form of the posttranscriptional modification and plays a critical role in many cellular processes (26). UCH-L3 is also believed to inhibit the degradation of various tumor-growth-promoting proteins by removing the trigger for degradation (27).

1.3.8 FAM

FAM is also known as Usp9X (28). It is an UBP enzyme of approximately 290 kD (2547 amino acids (29)) and it is the human homolog of the *Drosophila melanogaster fat facets (faf)* gene product (28,28,30). FAM interacts with the adhesion and signaling molecule AF-6 at cell-cell contact sites in epithelial cells. AF-6 serves as one of the peripheral components of cell-cell adhesions, and is thought to participate in the regulation of cell-cell adhesions downstream of Ras. FAM prevents the ubiquitination of AF-6. Thus, the degradation of peripheral components of cell-cell adhesion such as AF-6 appears to be regulated by FAM (30,31).

FAM also interacts with ß-catenin. ß-catenin establishes a link with the actin cytoskeleton in cell-cell adhesions by forming complexes with cadherin and α-catenin (31). The cadherin-catenin complexes are important for the maintenance of epithelial architecture (32). ß-catenin is also involved in nuclear signal transduction. In the cytoplasm, the Wnt signaling pathway regulates the stabilization of ß-catenin. The activation of this pathway results in an inactivation of glycogen

synthase kinase-3ß (GSK-3ß), and leads to the accumulation of ß-catenin in the cytoplasm and its translocation into the nucleus. In the absence of a Wnt signal, GSK-3ß is thought to phosphorylate ß-catenin and subsequently to induce its degradation. The ubiquitin-proteasome pathway regulates the degradation of ß-catenin. The phosphorylated ß-catenin is ubiquitinated and degraded by the proteasome. It has been shown that FAM interacts with ß-catenin, so it is possible that FAM regulates the degradation of ß-catenin by preventing its ubiquitination. The FAM-binding site of ß-catenin maps to the region that is close to the APC or Axin-binding site of ß-catenin. This raises the alternative possibility that FAM inhibits the binding of APC and/or Axin to ß-catenin and that it represses the function of APC and/or Axin. It has been shown that overexpression of FAM results in an elevation of ß-catenin levels and in an elongation of the half-life of ß-catenin (31) (Figure 1.3.1).

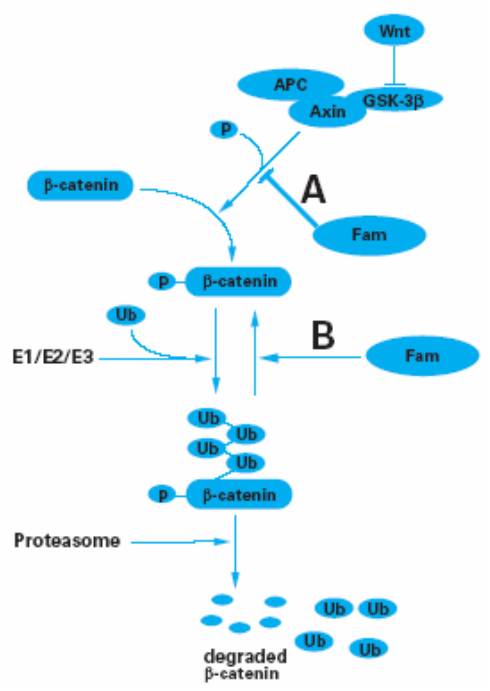

Figure 1.3.1: Model for the stabilization of ß-catenin by FAM. In the absence of the Wnt signaling pathway, various molecules such as GSK-3ß, APC or Axin promote the phosphorylation of ß-catenin. The phosphorylated ß-catenin is then ubiquitinated and degraded by the proteasome. FAM stabilizes the ß-catenin. There are two hypotheses on how this works: (A) FAM may inhibit the binding of APC and/or Axin to ß-catenin and repress the function of APC or Axin. (B) FAM may release Ub directly from the ubiquitinated ß-catenin. Adapted from (31).

The genes activated by ß-catenin are involved in fundamental developmental and cell biological processes, such as cell proliferation, differentiation and cell polarity. Dysregulation of the pathway can be detrimental, because several components are tumorigenic when mutated and are associated with hepatic, colorectal, breast and skin cancers (33). It has been shown that ß-catenin is involved in apoptosis, it has an important role at a late stage of apoptosis (34).

1.4 The involvement of the ubiquitin system in the cell cycle

1.4.1 The E3 ubiquitin ligase BRCA1

The product of the hereditary breast cancer gene BRCA1 is expressed in late G1/S-phase of the cell cycle, where it forms foci at sites of DNA replication (35) (Figure 1.4.1).

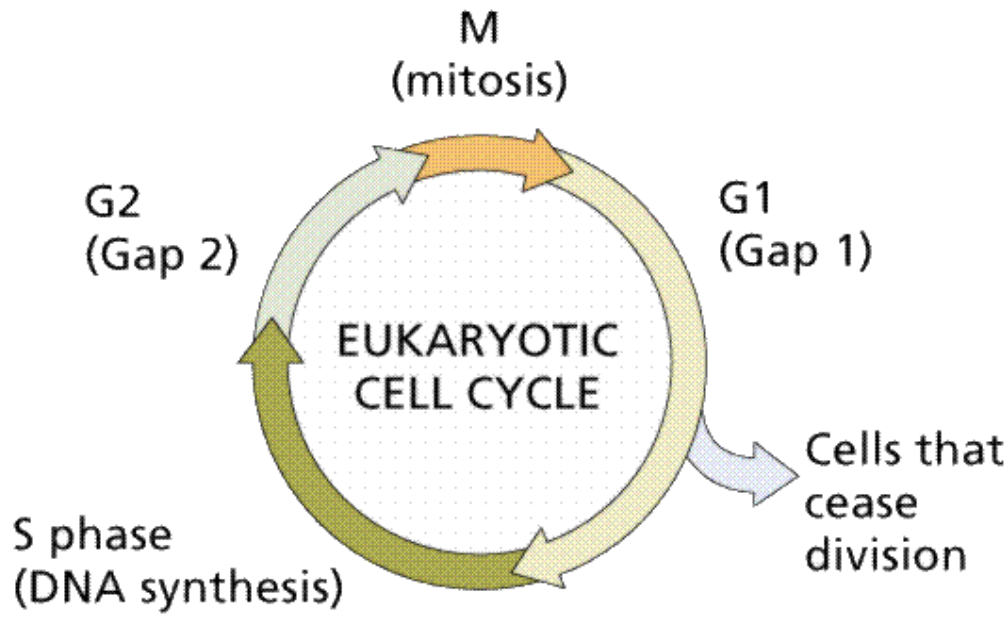

Figure 1.4.1: A schematic presentation of the eukaryotic cell cycle. Adapted from (36).

BRCA1 is also recruited to sites of DNA damage following ionizing radiation or replication block by hydroxyurea (HU) where it colocalizes with many DNA repair proteins. It has been shown that loss of BRCA1 results in sensitivity to genotoxins in mouse and in human cells, so BRCA1 appears to be important for cellular responses to DNA damage. Moreover, cells deficient for

BRCA1 exhibit defective repair of double-stranded breaks in S-phase by homologous recombination (35).

The N-terminus of the BRCA1 protein bears a RING finger domain that functions as an E3 ubiquitin ligase in vitro. The ability of BRCA1 to synthesize monoubiquitinated and polyubiquitinated proteins is greatly increased when it is in a complex with its N-terminal binding partner BARD1. Down-regulation of endogenous, cellular BRCA1:BARD1 results in abrogation of ubiquitin conjugation in DNA replication structures in S-phase and following treatment with hydroxyurea (HU) or ionizing radiation, suggesting that heterodimer activity is required for their formation. Conjugation of ubiquitin in foci is inhibited by the expression of ubiquitin bearing a lysine 6 mutation suggesting that the ubiquitin polymers formed at these sites are dependent on lysine-6 for linkage. BRCA1 directed ligation of ubiquitin occurs during S-phase and in response to replication stress and DNA damage and is therefore likely to be a significant aspect of BRCA1 cellular activity (35).

Individuals who carry mutations in the BRCA1 gene are predisposed to early onset breast and ovarian cancer. The precise function of BRCA1 in tumor suppression remains speculative, but the protein has been implicated in many varied cellular function including DNA repair and cell cycle check-point control, centrosome duplication and transcription (35).

1.5. Some diseases where ubiquitin might be involved

1.5.1 Inflammatory bowel disease

Inflammatory bowel disease (IBD) is a term used to describe a collection of diseases that involve the bowel and are characterized by the production of inflammation and at times ulceration in the small or large bowel. The two most common disorders in this group are ulcerative colitis (UC) and Crohn's disease (CD) (37,38). The clinical diagnosis of UC and CD may overlap, and as a result of this a diagnosis of intermediate colitis (IC) is made (39). IBD most commonly begins during adolescence and early adulthood. The cause is yet unknown, but it is probably multifactorial. There is evidence of a genetic link in IBD. Jewish people are at increased risk of developing the condition,

while African Americans are at decreased risk (38,40). It has also been shown that the environment has a big influence on the onset of the disease (41).

UC refers to ulceration of the colon that has no known cause. In this condition it is commonly only the lining of the bowel that is inflamed. It has recently been shown that the level of the tumor suppressor protein p53 is increased in UC (42).

CD is named after the physician who first described the condition. The two primary sites for CD are the ileum, which is the last portion of the small bowel, and the colon, but it can affect the digestive system anywhere from the mouth to the anus (43-45). The inflammation tends to involve the entire bowel wall, so it is more extensive than in UC (38,46). In the early stages CD causes small, scattered, shallow, crater-like areas on the inner surface of the bowel. With time, these erosions become deeper and larger, ultimately becoming ulcers that cause scarring and stiffening of the bowel (44,45) (Figure 1.5.1).

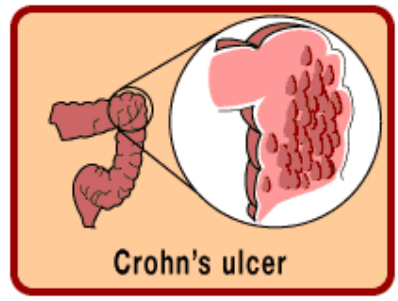

Figure 1.5.1: Illustration of the ulcers in Crohn's disease. Adapted from (44).

As the disease progresses, the bowel becomes increasingly narrowed and it can ultimately be obstructed. The small intestine is much narrower than the colon, so obstruction of this part of the digestive system is most likely (45) (Figure 1.5.2).

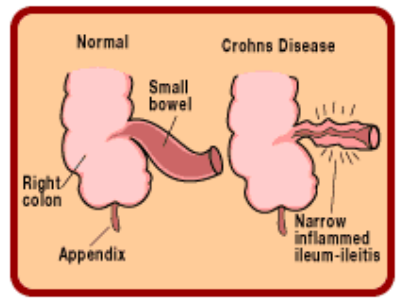

Figure 1.5.2: The difference between a normal small bowel and the narrowed small bowel in Crohn's disease. Adapted from (44).

Deep ulcers can puncture holes in the wall of the bowel, and bacteria from within the bowel can spread to infect adjacent organs and the surrounding abdominal cavity (45).

The most common symptom of IBD is diarrhea. Other symptoms include cramping, abdominal pain, fever, joint pains and skin lesions (38,46). The symptoms and severity of IBD vary among patients, and each individual patient will typically experience periods of relapse followed by periods of remission lasting months to years (2,45). The development of fistulas, abscesses and intestinal obstruction is most commonly found in CD, but it may also appear in UC. Patients with IBD also have a higher risk of bowel cancer. This is most often seen in patients with a long history of UC but may also be seen in patients with CD. In addition, in CD there is a higher risk of small bowel cancer (38,46). There is no medical cure for IBD, but there are some drugs that can ease the pain and promote remission, including anti-inflammatory drugs, immunosuppressive drugs and antibiotics. Diet is an important factor in the management of IBD. Surgery is also commonly used at some point in IBD (38,46).

Transforming growth factor ß (TGF-ß) might have a role in the pathogenesis of IBD. It is a potent endogenous downregulator of inflammation, made by virtually all cell types. Mice in which the TGF-ß1 gene has been deleted die of generalized inflammation early in life, a component of which is a severe colitis. The level of TGF-ß is markedly increased in IBD tissues. Signals from the TGF-ß receptor to the nucleus are transmitted through a cascade of proteins termed Smads. As well as signal transducing Smads, there are also inhibitory Smads whose function is to downregulate

TGF-ß signaling inside cells. It has been shown that an inhibitory Smad, Smad7, is overexpressed in CD mucosa and that when cells from CD patients are stimulated with TGF-ß there is no activation of the signaling cascade. Although TGF-ß potently dowregulates proinflammatory cytokine production it has no effect on cytokine production by cells from IBD patients. The control of Smad7 in gut of IBD patients is not known, but its presence clearly is important in whether a cell is able to respond to TGF-ß. A quantitative analysis of Smad7 RNA has revealed no difference between IBD and normal intestinal samples. This and other findings suggest a great complexity in the regulation of Smad7 and that in IBD, Smad7 is regulated at the post-transcriptional level (41,47).

1.5.2 Ischemic heart disease

The medical term for this condition is ischemic heart disease (IHD), but the common name is coronary artery disease. The disease is characterized by decreased supply of blood to the heart because the coronary arteries are narrowed or blocked due to deposition of atheromatous plaques on their walls (Figure 1.5.3).

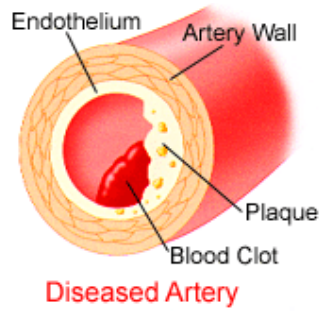

Figure 1.5.3: A diseased coronary artery with plaque and blood clot. Adapted from (48).

These fatty deposits build up gradually and irregularly in the large branches of the two main coronary arteries that encircle the heart and are the main source of its blood supply. The decreased supply of blood reduces the supply of oxygen and nutrients to the heart musculature, which is essential for proper functioning of the heart. This ischemia and deprivation of nutrients may cause

damage to the heart muscle, and complete occlusion of the blood vessel leads to the death of that area of heart tissue, which may lead to infarction (49) (Figure 1.5.4).

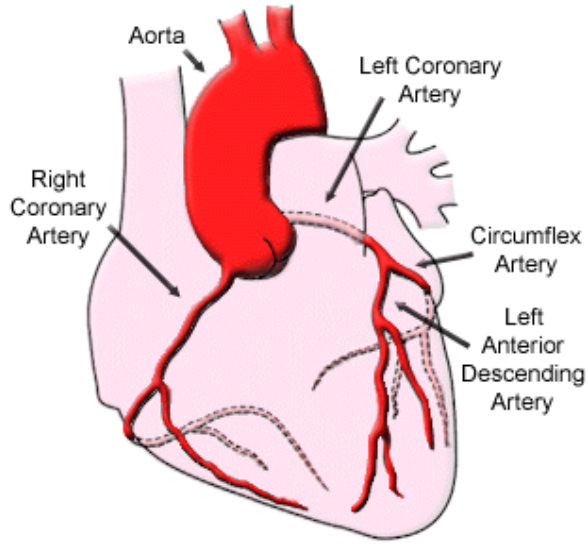

Figure 1.5.4: A figure of the heart showing the branches of the two main coronary arteries that encircle the heart. Adapted from (48).

There are a number of risk factors for developing IHD, including smoking, hypertension, diabetes mellitus and hypercholesterolemia. Genetic factors may also be involved (50).

There are no symptoms in the early stages of IHD. At later stages the first sign is often angina pectoris, which is typically precipitated by physical activity and relieved by rest. The chest pain is usually worse after a heavy meal and with exposure to cold air. The pain is located over the central chest and may sometimes radiate down the left arm, to the jaw or to the back. Some patients have silent ischemia, that is, they have IHD without experiencing the angina. For these patients the first sign of IHD may be the severe chest pain of a heart attack (49).

As the heart supplies oxygenated blood to the various organs of the body, any defect in the heart immediately affects the supply of oxygen to the vital organs such as the brain and kidneys. This may lead to the necrosis of tissue within these organs and their eventual failure (50).

There is currently no cure for IHD, but with proper treatment most patients will be able to lead normal and healthy lives. Treatment is usually with drugs and also involves advice regarding

regular exercise, avoiding smoking, diet control and life style modification. A minority of patients requires surgical therapy. The preferred procedure is then coronary angioplasty, were the blocked vessel is dilated by inflating a balloon inside the vessel. Another option is coronary artery bypass grafting, were the blocked area of the vessel is replaced using a graft from the patient (50).

The exact causes of IHD are yet unknown. Unidentified factors initiate the formation of atheromatous plaques in the coronary arteries, which slowly lead to ischemic conditions for the affected cells. The ischemia leads to rapid changes in myocardial metabolism, the degree of which is highly dependent upon the severity of the ischemia. The inadequate oxygenation may cause transient disturbances of the mechanical, biochemical, and electrical functions of the myocardium. When oxygenated, the normal myocardium metabolizes fatty acids and glucose to carbon dioxide and water. With severe oxygen deprivation, fatty acids cannot be oxidized, and glucose is broken down to lactate. This reduces the intracellular pH and the myocardial stores of high-energy phosphates like adenosine triphosphate (ATP). Impaired cell membrane function leads to potassium leakage and the uptake of sodium by cardiac myocytes. The severity and duration of the ischemia will determine whether the damage is reversible or whether it is permanent. Permanent damage leads to cardiac myocyte cell death by apoptosis, and is a central feature of IHD. High levels of apoptosis are evident in the myocardium of failing human hearts and in association with myocardial infarction, both within the infarcted area itself and in the surrounding viable tissue. The ischemia activates apoptosis of the cardiac myocytes through at least three distinct pathways involving reperfusion, energy depletion, and acidosis (50-53).

Some findings suggest that the ubiquitin system might have a role in IHD. The ubiquitin system has important functions in various biological pathways including inflammation, cell proliferation and apoptosis, all of which constitute important characteristics of the formation of atheromatous plaques. For example, the transcription factor NF-κB has been identified as an important mediator in the inflammatory-proliferative process generating atheromatous plaques. The ubiquitin-proteasome system is involved in the activation of NF-κB, although the necessity of the involvement of this system in NF-κB activation, particularly under conditions of aggravated oxidative stress, has been questioned (54). Another example concerns a study on pigs, which

demonstrated accumulation of ubiquitin-conjugates in coronary arteries after a 12-week high-cholesterol diet. In this experiment the proteolytic activity of the proteasome remained functionally unimpaired. Supplementation of the high-cholesterol diet with vitamin C and E was associated with a decrease in the amount of ubiquitin-protein conjugates, indicating that the increase in ubiquitination in the coronary arterial wall was oxidation-sensitive. The reason for this accumulation of ubiquitin conjugates remains to be established (55).

Increase in ubiquitinated products has been observed in a variety of pathophysiologic states associated with increased oxidative stress. Despite this association with enhanced oxidative stress, reports on the expression of ubiquitin and ubiquitin-protein conjugates in atherosclerotic cardiovascular disease have remained scarce (55). From what can be gathered from the very few published studies on the ubiquitin-proteasome system in cardiovascular diseases, including IHD, the system seems to be functionally active to a different extent in the initiation, progression and complication stage of atherosclerosis. These early findings need further attention and confirmation and only time will tell the precise roll of the ubiquitin system in these diseases (54).

1.5.3 Dilated cardiomyopathy

Dilated cardiomyopathy (DCM) is a disease of the heart muscle leading to an enlarged heart that pumps blood less strongly. The reasons why the heart muscle weakens are in the majority of cases unknown. In a few cases however, there are some factors that are thought to cause or contribute to the disease. Examples of these factors are mild genetic abnormalities, viral myocarditis caused mainly by Coxsackie virus, autoimmune disease of the heart, excessive alcohol consumption and pregnancy. The process of developing DCM is probably slow and the disease becomes symptomatic only when quite advanced (56) (Figure 1.5.5).

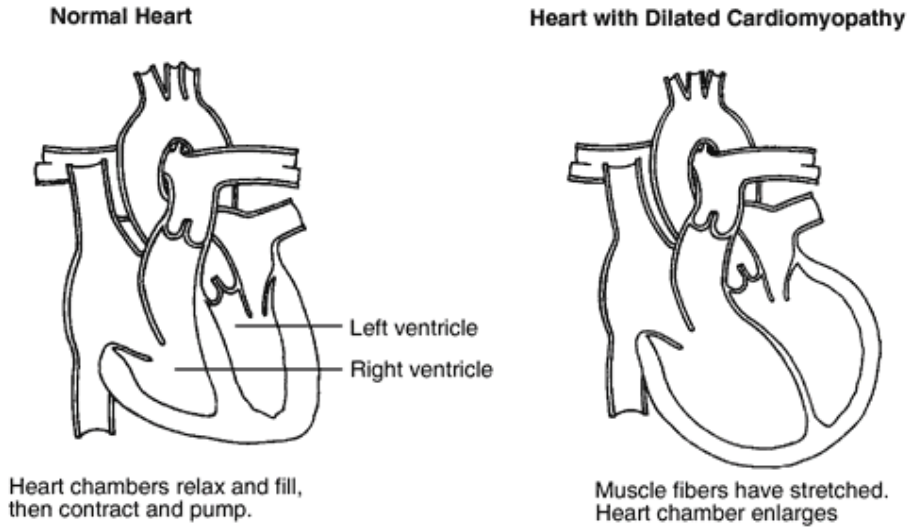

Figure 1.5.5: Comparison of a normal heart and a heart with dilated cardiomyopathy. Adapted from (57).

Common diseases like coronary heart disease, hypertension and heart valve disease also causes a dilated heart. In DCM however, all these other causes have, by definition, been out ruled, and the disease is caused by an intrinsic heart muscle problem (56).

The symptoms of DCM differ with the stages of the disease. The symptoms may come on slowly or can be very sudden in onset. When the heart muscle becomes weak and is unable to pump blood efficiently, fluid builds up in the lungs, which then become congested. This result in a feeling of breathlessness and a condition called left heart failure. Breathlessness is very common. Some patients experience this symptom only when they exert themselves, while others are breathless even at rest (56). Often DCM also involves right heart failure. This causes oedemas in the legs and ankles and accumulation of fluid in the liver and abdomen. Some only have mild oedema towards the end of the day, while people with more severe disease have lot of oedema all the time (56). Atrial fibrillation is very common in DCM. The heartbeat is irregular and rapid and causes symptoms of palpitations, increasing shortness of breath and fatigue. The reason for fatigue is that because the heart is weak, limb muscles do not receive an adequate supply of blood, especially when exercising and this may lead to a feeling of tiredness. The atrial fibrillations can be associated with sudden deterioration of symptoms or the development of blood clots (56). Since the blood flow through the heart is slower then normal in patients with DMC, blood clots may be allowed to

form within the heart. If a blood clot dislodges from these sites and floats into circulations it can eventually lead to a stroke (56). Ventricular tachycardia, a very fast heart beat, occurs quite commonly in DCM. It is often associated with a fall in blood pressure, and symptoms of dizziness, breathlessness or fainting, but can be symptomless (56). DMC may in rear cases lead to sudden death, usually caused by a severe arrhythmia or the development of a large blood clot (56). There is currently no cure for DCM, although some patients improve spontaneously. Treatment is usually with drugs and is aimed at minimizing symptoms and preventing the development of complications and progression of the disease. A minority of patients deteriorates in spite of treatment and may require cardiac transplantation (56).

The pathological mechanisms and molecular causes of DCM have not yet been determined, but it might seem that the ubiquitin system is somehow involved. It has been shown that DCM hearts have significantly higher total protein-ubiquitin conjugation compared to control donor heart tissues, and some of these proteins are ubiquitinated only in DCM hearts (58). DCM hearts also show significantly higher expression of certain key enzymes of the ubiquitin-proteasome pathway. For example, the expression of ubiquitin C-terminal hydrolases (UCH) is increased and the expression of the E1 and E2 enzyme components of the ubiquitin-proteasome system is also increased in DCM hearts compared to controls (58).

Coxsackie virus is the primary causative agent of viral myocarditis, which again leads to DCM. It has been shown that infections with these viruses facilitate the ubiquitin-proteasome processing of a protein involved in the cell cycle and the tumor suppressor p53. This raises the possibility that the ubiquitin-proteasome pathway may be used by the virus to promote its replication. It has been shown that treatment of murine cardiomyocytes with proteasome inhibitors significantly decreases the virus load and prevents virus-induced cell death. Inhibition of the ubiquitin-proteasome pathway does not affect virus entry, but the viral RNA transcription and protein translation are markedly reduced. However, ubiquitin-proteasome pathway-mediated viral replication does not appear to be related to changes in proteasome activities (59).

Only time will tell the exact role of the ubiquitin system in DCM, but it is evident that any change in the expression, degradation or activity of components in this system would have profound implications to the cell, tissue or organ in which this occurs.

1.6 The nuclear factor-κB pathway

1.6.1 Nuclear factor-κB

The nuclear factor-κB (NF-κB) proteins are a family of ubiquitously expressed transcription factors that, in mammals, consist of five members: p65 (RelA), RelB, c-Rel, NF-κB1 (p50 and its precursor 105) and NF-κB2 (p52 and its precursor p100). These proteins exist in virtually all eukaryotic cell types. Dimerization of the NF-κB family members is necessary for their DNA-binding properties. Both homodimeric and heterodimeric combinations are possible. The p65-p50 heterodimer was defined as the classical NF-κB binding form, but this is only one of the multiple species that can bind to κB sites. The most prevalent activated form of NF-κB is a heterodimer consisting of a p50 or p52 subunit and p65. The diverse regulation of NF-κB-dependent promoters is, at least in part, due to the ability of different dimers to bind to the same or distinct κB sites in a cell-type- and stimulus-dependent manner (4,60).

The NF-κB pathway can be activated by a variety of stimuli including cytokines, bacterial or viral infection and stress. Because of the presence of different activating signals, NF-κB proteins and regulatory proteins, there are different versions of the NF-κB pathway. The detailed mechanisms behind all these different pathways are still unclear, but they all finally result in the activation of the NF-κB-dependent promoters (4).

In most unstimulated cells, the NF-κB proteins are predominantly localized in the cytoplasm where they are bound to a family of inhibitory proteins known as Inhibitor-κB (IκB), of which the most important may be IκBα, IκBß and IκBε. IκBα is associated with transient NF-κB activation, whereas IκBß is involved in sustained activation. Hence, different IκB molecules have distinct, and sometimes overlapping, specificities. The tissue distribution may also differ for various IκBs. Upon receipt of an appropriate signal, the NF-κB proteins are released from the IκB proteins and translocate to the nucleus where they can upregulate transcription of specific genes. The ubiquitin

system is involved in the mechanism behind this release. The activation of the NF-κB pathway induces the phosphorylation of IκB proteins. Increased phosphorylation of IκB by the IκB kinases results in the ubiquitination and proteasomal degradation of IκB and hence the nuclear translocation of the liberated NF-κB protein. Although the primary function of IκBα is to retain the NF-κB proteins in the cytoplasm of unstimulated cells, it is also involved in the removal of NF-κB proteins from the nucleus (4,60) (Figure 1.6.1).

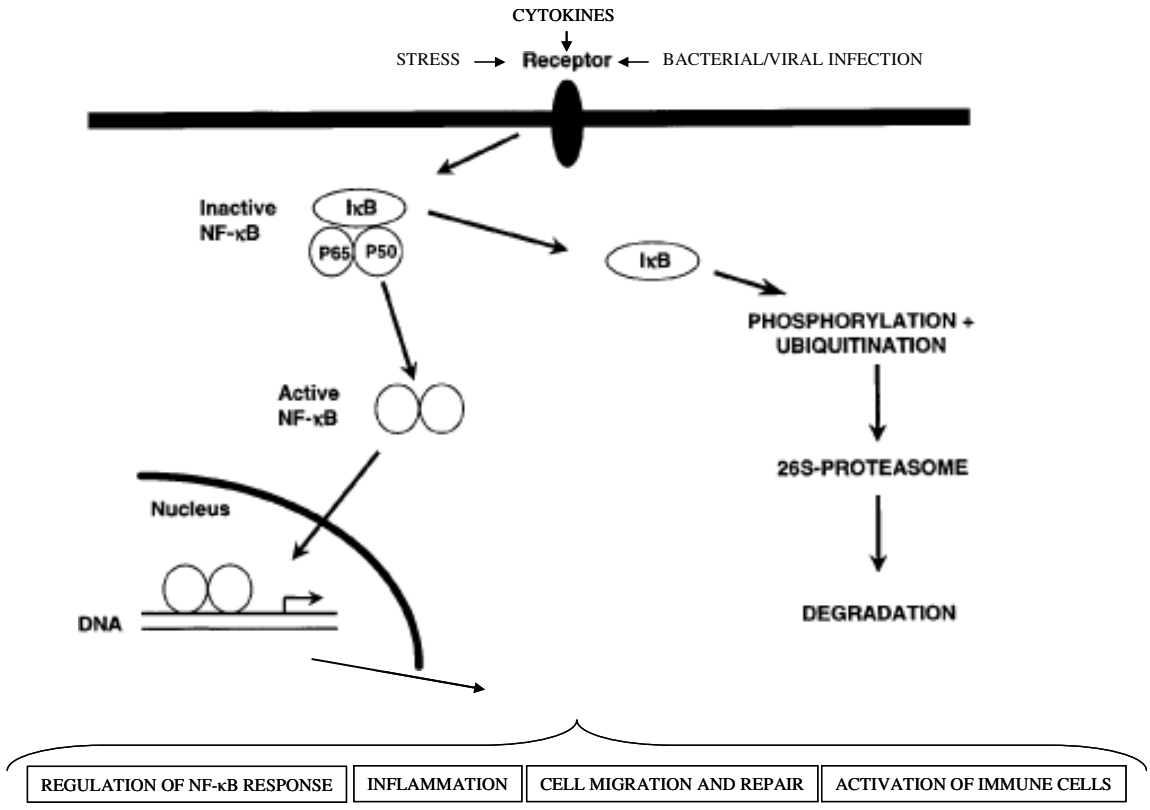

Figure 1.6.1: Simplified mechanism for induction of NF-κB proteins. Adapted from (61) and modified by the help of (62).

NF-κB is clearly one of the most important regulators of proinflammatory gene expression. The NF-κB pathway is involved in increasing the expression of crucial molecules that are important in the regulation of cellular proliferation, apoptosis and cell-cycle progression (60).

In some cells the activity of the IκB kinases is greatly enhanced by tumor necrosis factor α (TNF-α) and interleukin 1 (IL-1), leading to degradation of endogenous IκBα and nuclear translocation of NF-κB (4). It has been demonstrated that NF-κB in certain situations acts as an anti-apoptotic

protein. Strong support for a protective role for NF-κB in apoptosis came from studying the effects of TNFα on cell killing. The cytokine TNFα was initially characterized by its ability to kill tumor cells. However, it turned out that its ability to kill cells was highly cell-type dependent. Reports indicated that inhibiting NF-κB activity in cells made cells that were otherwise insensitive to TNF-mediated killing exquisitely susceptible to apoptosis. It also turned out that this protective function of NF-κB also was apparent on cells treated with ionizing radiation or chemotherapeutic agents. These findings suggested that NF-κB may play an anti-apoptotic role in many systems. Later studies have reviled that the role of NF-κB in apoptosis is not always straightforward. NF-κB activation may lead to induction of apoptosis in some cell types while in other lineages NF-κB plays an antiapoptotic role. It appears that NF-κB under certain circumstances serves to upregulate the synthesis of one or many antiapoptotic gene products (4,60,62).

In addition to the inducible genes, NF-κB has been found in an active, nuclear form in mature B cells, plasma cells, macrophages, and some neurons. Constitutive activation of the NF-κB pathway is among other tings associated with inflammatory and autoimmune diseases, such as, atherosclerosis, IBD and cancer (60).

The role of NF-κB in modulating the expression of so many different cytokines and lymphokines strongly supports its proposed role as a coordinating element in the body's response to situations of stress, infection, or inflammation. Therefore, development of specific inhibitors of its function should lead to novel therapeutics, which may serve to effectively treat situations such as inflammation (62).

1.7 Prior experiments

Before I joined the Intracellular Signaling Laboratory, Imperial College, my supervisor and co-workers had used thiol-reactive, ubiquitin probes to identify several tissue-specific DUBs. They had found that thymus contains an ubiquitin protease of approximately 64 kD that is expressed at particularly high levels. This protease was also detected at low levels in lymph nodes, spleen, bone marrow and other tissues. The most interesting findings were generated when they analyzed the activity of DUBs in proliferating T-cells compared to quiescent cells. The T-cells were induced

through cognate binding of T-cell receptors to peptide/MHC complexes. Two DUBs were specifically activated in proliferating T-cells. Combined with probe these proteins were detected at 60 kD and 70 kD respectively. Both enzymes were also detected at low levels in thymus, lymph node and spleen. The identity of either molecule was unknown.

Since the activity of these two DUBs was elevated greatly during T-cell proliferation in response to antigen, it was suggested that these molecules may regulate the development of T-lymphocytes or their subsequent responses to antigen.

1.8 Aims of the study

The aims of the present study are to:

1. *Investigate whether the ubiquitin hydrolase of 60 kD or 70 kD (approximately 50 kD and 60 kD without the probe respectively) is also found in human umbilical vein endothelial cells (HUVEC) and if one of these DUBs corresponds to the UBP DUB-2.*

2. *Study the expression of DUBs and the levels of ubiquitinated enzymes in different stages of the cell cycle.*

3. *Obtain tissues from patients with inflammatory bowel disease (IBD), ischemic heart disease (IHD) and dilated cardiomyopathy (DCM) and analyze them for differences in ubiquitin levels and ubiquitin hydrolases.*

My preliminary studies generated two additional aims:

4. *Examine whether the expression of DUBs in endothelial cells is regulated by cell confluence, culture time or hypoxia.*

5. *Investigate the effect of hypoxia on the translocation of NF-κB into the nucleus.*

2. MATERIALS AND METHODS

2.1 Preparing reagents for experiments

2.1.1 Preparation of TBE buffer (10x)

109 g Tris (Tris(hydroxymethyl)aminomethane (Fluka)), 55 g Boric acid (Borax (Sigma)) and 20 ml 0.5 M EDTA (Etylenediamineteraacetic acid (Sigma)) was added to a final volume of 1 L.

2.1.2 Preparation of lysis buffer

A 500 ml stock solution of 50 mM Tris (Tris(hydroxymethyl)aminomethane (Fluka)), 250 µM sucrose (Sucrose (BDH)) and 1 mM $MgCl_2$ (Sigma) was prepared and the pH was adjusted to 7.4 by using a pH-meter (Corning pH meter 240 (Meadowrose scientific LTD)). 1 mM DTT (DL-Dithiothreitol (Sigma)), 0.5 mM AEBSF (4-(2-Aminoethyl)benzenesulphonyl fluoride hydrochloride (Sigma)) and 0.1% NP40 (Nonidet®P 40 substitute (Sigma)) were added just before using the lysis buffer.

2.1.3 Preparation of reaction buffer

A 100 ml solution of 50 mM HEPES (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (Sigma)) and 0.5 mM EDTA (Etylenediamineteraacetic acid (Sigma)) was made, and the pH was adjusted to 7.8.

2.1.4 Preparation of Coomassie blue destaining solution

This comprised 500 ml methanol (Methanol microscopy (BDH)), 400 ml ultra pure water and 100 ml acetic acid (Acetic acid ~100% AnalaR® (BDH)).

2.1.5 Preparation of LB-medium

5 g NaCl (Sodium Chloride AnalaR® (BDH)), 5 g Tryptone (Bacto™Tryptone (BD)), 2.5 g Yeast extract (Yeast Extract (DIFCO)) and 6 g Bacto agar (Bakto™Agar (DIFCO)) were mixed and

ultra pure water was added to a final volume of 500 ml. The solution was mixed well on a stirrer and the pH was adjusted to 7.0. The solution was sterilized by autoclaving.

2.2 Isolating, growing and passaging HUVEC

2.2.1 Complete medium for HUVEC

100 ml heat inactivated FCS (fetal calf serum (Sigma)) was added to 390 ml M199 (Medium 199 with Earle's salts and $NaHCO_3$, without L-glutamine (Sigma)) after sterile filtration 0.20 µm. 5 ml L-glutamine (L-glutamine solution 200 mM (Sigma)) and 5 ml pen/strep (10,000 units/ml penicillin and 10 mg/ml streptomycin in 0.9% sodium chloride (Sigma)) were added by filtration 0.20 µm. When added to cells, 10 µl/ml of ECGS (Endothelial cell growth supplement from bovine neural tissue (Sigma) dissolved in 1000 I.V./ml Heparin (Monoparin)) was added to complete the medium.

2.2.2 Collection of umbilical cords and isolation of HUVEC

Umbilical cords were collected in a pot containing 100 ml HBSS with Ca/Mg (Hanks balanced salt solution, modified, with $NaHCO_3$, with Ca/Mg (Sigma)), 1 ml gentamycin (Gentamycin sulfate salt hydrate (Sigma)) and 1.2 ml Na pyruvate (Invitrogen). Umbilical cords were used up to one day old.

In sterile conditions, a 25 cm^2 flask (BD Falcon™) was coated with 1.5 ml 1% gelatin (Sigma) and incubated at 37°C (Galaxy CO_2 incubator (RS Biotech), temp: 37°C, CO_2: 5.0%). The cord was removed from the pot using forceps. The cord was held in one end with a piece of paper towel soaked with 70% ethanol (VWR) while another piece of soaked paper towel was used to squeeze down the length of the cord, cleaning the surface of the cord while also squeezing out any blood from inside. The cord was checked for large holes and clamp marks which were sealed using crocodile clips. A clean scalpel was used to cut the cord 3 cm from one end and the vein and the two arteries were located. The vein was opened up using forceps and a 3-way tap (BD Connecta™ Plus3 (BD)) was inserted into it and secured by tying with thread. The lumen of the umbilical vein was washed using 20 ml HBSS without Ca/Mg (Hanks balanced salt solution, modified, with

$NaHCO_3$, without Ca/Mg (Sigma)) (37°C) which was introduced using a syringe fitted to the 3-way tap. The cord was then cut 3 cm from the other end and another 3-way tap was inserted and tied firmly. The umbilical vein was filled with 20 ml HBSS without Ca/Mg containing 100 µl collagenase (100 mg/ml Collagenase (Sigma)) and the cord was put on a small tray, covered with foil and put into the 37°C incubator for 8 minutes. The cord was returned to the hood and gently massaged on the outside. All the HBSS without Ca/Mg was squeezed into one syringe and emptied into a 50 ml Falcon tube (BD Falcon™ 50 ml (BD)). The vein was then flushed using 10 ml HBSS without Ca/Mg. This wash was added to the Falcon tube which was spun at 1200 rpm for 8 minutes (FunctionLine Labofuge 400R (Heraeus instruments)). The supernatant was discarded and the cell pellet was suspended in 5 ml complete HUVEC medium.

The gelatin was removed from the 25 cm^2 flask by using a vacuum pump (Medical suction high vacuum-high flow SAM12 (Abbey Surgical)) and the flask was washed with 5 ml HBSS without Ca/Mg. The cell suspension was added and the flask was incubated at 37°C. The following day the medium was changed to remove red blood cells and debris. When the cells were fully confluent they were passed on to a 75 cm^2 flask using 1 ml Trypsin/EDTA (Trypsin/EDTA solution 1x (Sigma)) (See section 2.2.3 Passaging HUVEC).

2.2.3 Passaging HUVEC

At 60-80% confluence, usually every 2-3 days, the cells were split to maintain them in a proliferative state. The cells could be used for experiments up to passage four. 75 cm^2 flasks (BD Falcon™) were coated with 2.5 ml 1% gelatin (Sigma) 20 minutes prior to passing the cells. 50 ml complete HUVEC medium was pre-warmed to 37°C. The old medium was removed from the confluent 75 cm^2 flask by using a vacuum pump. 10 ml 37°C HBSS without Ca/Mg (Hanks balanced salt solution, modified, with $NaHCO_3$, without Ca/Mg (Sigma)) was added and the flask was rocked to wash the monolayer and then removed using vacuum pump. 2.5 ml Trypsin/EDTA (Trypsin/EDTA solution 1x (Sigma)) was then added and the flask was rocked to cover the monolayer before it was incubated at 37°C for 2-3 minutes. The flask was tapped firmly to release all the cells and a microscope (Leica DM16 (Leica)) was used to confirm that all the cells had loosened from the gelatin. The cells were resuspended in 17.5 ml fresh complete HUVEC medium

to quench the trypsin. The gelatin was removed from the new flasks before they were washed with 5 ml HBSS without Ca/Mg. The cell suspension was divided equally among three 75 cm^2 flasks and complete HUVEC medium was added to a total volume of 15 ml in each flask. The cells were incubated at 37^oC.

2.3 Thawing, growing and passaging HEK 293T cells

2.3.1 Medium for HEK 293T cells

100 ml FCS (fetal calf serum (Sigma)) was added to 390 ml DMEM (D-MEM (1x), liquid – with sodium pyruvate (Invitrogen)) after sterile filtration 0.20 µm. 5 ml L-glutamine (L-glutamine solution 200 mM (Sigma)) and 5 ml pen/strep (10,000 units/ml penicillin and 10 mg/ml streptomycin in 0.9% sodium chloride (Sigma)) were filtered 0.20 µm and added to complete the medium.

2.3.2 Thawing and establishing cultures of HEK 293T cells

An ampoule of HEK 293T cells was collected from the liquid nitrogen container and thawed rapidly at 37^oC. The cells were carefully resuspended in a 25 cm^2 flask (BD Falcon™) containing 5 ml complete medium equilibrated to 37^oC. The medium was changed the next day. When the cells were fully confluent they were passed on to a 75 cm^2 flask using 1 ml Trypsin/EDTA (Trypsin/EDTA solution 1x (Sigma)) (See section 2.3.3 Passaging HEK 293T cells).

2.3.3 Passaging HEK 293T cells

At 60-80% confluence, usually every 2-3 days, the cells were split to maintain their proliferative state and to expand cultures. The required volume of complete medium was pre-warmed to 37^oC. The old medium was removed from a 75 cm^2 flask with confluent HEK 293T cells by using a vacuum pump. 10 ml HBSS without Ca/Mg (Hanks balanced salt solution, modified, with $NaHCO_3$, without Ca/Mg (Sigma)) was added and the flask was rocked to wash the monolayer. The HBSS without Ca/Mg was removed using vacuum pump. 2.5 ml Trypsin/EDTA (Trypsin/EDTA solution 1x (Sigma)) was added to the cells and the flask was rocked to cover the monolayer before it was incubated at 37^oC for 2-3 minutes. The flask was tapped firmly to release all the cells from

the bottom of the flask, and this was confirmed using a microscope. The cells were resuspended in 17.5 ml fresh complete medium and divided into three 75 cm^2 flasks (BD Falcon™). Complete medium was added to a total volume of 15 ml in each flask. The cells were incubated at 37°C.

2.4 Preparation of plasmid DNA

2.4.1 Preparation of Petri dishes with selective LB-medium

A flask with autoclaved LB-medium (see section 2.1.5 Preparation of LB-medium) was heated in a microwave oven until the medium became soluble. The solution was cooled down to a temperature just above the melting point (about 40°C). In a sterile environment 1 µl/ml ampicillin (Sigma) was added to the solution. 25 ml of the medium was carefully added to the desired number of Petri dishes (Sterilin) without making any bubbles in the medium. The dishes were left in the sterile environment until they were partially dried. The dishes could be used up to one month when stored at 4°C.

2.4.2 Transformation of bacteria by heat shock

We received two different plasmids with DUB-2 complementary DNA (cDNA) and one plasmid with DUB-2 cs mutant cDNA. In the mutant the active site had been inactivated by replacing the catalytic cysteine with an inactive serine unit. The plasmids all contained a cytomegalovirus immediate-early gene promoter, which enabled binding of the bacterial RNA polymerase and the subsequent transcription of the DNA. The open reading frame in each plasmid contained the desired form of the DUB-2 DNA and a sequence encoding the FLAG epitope.

To amplify the amount of DNA we transformed the three plasmids into *Escherichia coli* bacteria by using the heat shock procedure. By exposing the transformed bacterial culture to ampicillin, all cells except those which had been encoded by the plasmid DNA recombinant were killed, leaving a cell culture containing the desired recombinant DNA.

The bacteria strain used was XL1-blue super-competent cells (200249) (Stratagene). 30 µl of the bacteria suspension was added to a chilled 1.5 ml eppendorf tube (Appleton Woods) and put on ice. 2 µl ß-mercaptoethanol (2-Mercaptoethanol cell culture tested (Sigma)) was added to the bacteria

suspension and the tube was incubated on ice for 10 minutes. The tube was gently swirled every second minute. 2 µl of the DNA construct was added to the bacteria and the tube was incubated on ice for 30 minutes. The Soc buffer (Invitrogen) was preheated to 42°C in a water bath. The bacteria suspension was heat pulsed in a water bath at 42°C for 45 seconds and incubated on ice for 2 minutes. 0.9 ml preheated Soc buffer was added to the eppendorf tube containing the bacteria and 100-200 µl of this mixture was spread on to Petri dishes containing LB-medium with ampicillin (see section 2.4.1 Preparation of Petri dishes with selective LB-medium).

2.4.3 MINI prep

To isolate the DNA from the bacteria we did a mini prep of the DUB-2 #2 cDNA. One 20 ml universal tube (Sterilin) per colony was filled with 5 ml LB medium containing 1 µl/ml ampicillin (Sigma) (see sections 2.1.5 Preparation of LB-medium). A single colony was picked from a freshly streaked selective plate by using a toothpick and the toothpick was inoculated in the medium. The tube was incubated for 8 hours at 37°C with vigorous shaking (~200 rpm) (SI-600R (Medline Scientific limited)). Bacterial solutions were transferred to eppendorf tubes and spun down at 13000 rpm (Eppendorf Centrifuge 5417R (Meadowrose scientific LTD)) for 7 minutes and the supernatant was removed. The pellet was spun down further at 13000 rpm for 30 seconds to remove the residual liquid. Plasmid DNA was extracted using a kit (QIAprep® Spin Miniprep kit (50) (QIAGEN)). The bacterial pellet was resuspended in 250 µl Buffer P1 (containing RNase A) and transferred to a microcentrifuge tube. 250 µl Buffer P2 was added to the bacterial suspension and the tube was gently inverted 4-6 times until the solution became viscous and slightly clear. 350 µl Buffer N3 was then added and the tube was immediately but gently inverted 4-6 times until the solution became cloudy. The tube was centrifuged for 10 minutes at 13000 rpm and a compact pellet was formed. The supernatant was applied to a QIAprep spin column by decanting. The column was centrifuged for 1 minute at 13000 rpm and the flow-through was discarded. The QIAprep spin column was washed by adding 0.75 ml Buffer PE and centrifuging at 13000 rpm for 1 minute. The flow-through was discarded and the column was centrifuged for an additional minute to remove residual wash buffer. The QIAprep column was placed in a clean 1.5 ml eppendorf tube and the DNA was eluted by adding 50 µl ultra pure water to the center of each QIAprep spin column. The column was let to

stand for 1 minute before centrifuging the tube at 13000 rpm for 1 minute. Plasmid DNA was quantified (see sections 2.4.5 Quantifying the amount of DNA by using a spectrophotometer) and analyzed by gel electrophoresis (see sections 2.4.6 Preparation and running of an agarose gel).

2.4.4 MIDI prep (QIAGEN Plasmid Purification)

To isolate the DNA from the bacteria we did midi preps of the DUB-2 cs cDNA and the DUB-2 #1 cDNA. Three 20 ml universal tubes (Sterilin) per sample were filled with 4 ml LB medium containing 1 µl/ml ampicillin (Sigma) (see sections 2.1.5 Preparation of LB-medium). By using a toothpick a single colony was picked from a freshly streaked selective plate and the toothpick was inoculated in the medium. The tubes were incubated for 8 hours at 37^{o}C with vigorous shaking (~200 rpm). The starter culture was transferred to a sterile 500 ml Erlenmeyer flask and diluted 1/500 using LB media containing ampicillin. For high-copy plasmids 25 ml media was inoculated while for low-copy plasmids 100 ml media was inoculated. The bacteria were grown at 37^{o}C for 12-16 hours with vigorous shaking (~300 rpm). The bacterial cells were harvested by centrifugation at 6000 rpm (RC 5C plus (SS-34) (Sorvall)) for 15 minutes at 4^{o}C. Plasmid DNA was extracted using a kit (QIAGEN® Plasmid Midi kit (25) (QIAGEN)). The bacterial pellet was resuspended in 4 ml Buffer P1 (containing RNase A). The bacteria solution was resuspended completely by pipetting up and down until no cell clumps remained. 4 ml Buffer P2 was added and the solutions were gently but thoroughly mixed by inverting 4-6 times before the tube was incubated at room temperature for 5 minutes after which the lysate appeared viscous. After use, the bottle containing Buffer P2 was closed immediately to avoid acidification from CO_2 in the air. 4 ml of chilled Buffer P3 was added and mixed immediately but gently by inverting 4-6 times and the tube was incubated on ice for 15 minutes. The addition of Buffer P3 led to the formation of a fluffy white material and the lysate became less viscous. The precipitated material contained genomic DNA, proteins, cell debris and SDS. The lysate had to be mixed thoroughly to ensure that even potassium dodecyl sulfate precipitated. More mixing was required until the mixture no longer appeared viscous and brownish to completely neutralize the solution. The mixture was centrifuged at 20000 rpm for 30 minutes at 4^{o}C and the supernatant containing the plasmid DNA was removed promptly. A QIAGEN-tip 100 was equilibrated by applying 4 ml Buffer QBT and then allowing the column to

empty by gravity flow. The supernatant containing the plasmid DNA was applied to the QIAGEN-tip and allowed to enter the resin by gravity flow. The QIAGEN-tip was washed two times with 10 ml Buffer QC. The DNA was eluted with 5 ml QF buffer and the DNA was precipitated by adding 3.5 ml isopropanol (BDH). The DNA was centrifuged at 15000 rpm for 30 minutes and the supernatant was carefully decanted. The DNA was washed by adding 2 ml 70% ethanol (Ethanol 99.7-100% v/v AnalaR® (BDH)) and then centrifuging at 15000 rpm for 10 minutes before the supernatant was carefully decanted without disturbing the pellet. The pellet was air dried for 5-10 minutes and redissolved in ES-buffer. Plasmid DNA was quantified (see sections 2.4.5 Quantifying the amount of DNA by using a spectrophotometer) and analyzed by gel electrophoresis (see sections 2.4.6 Preparation and running of an agarose gel).

2.4.5 Quantifying the amount of DNA by using a spectrophotometer

1 µl of each DNA sample was diluted in 99 µl ultra pure water. The spectrophotometer (Ultrospec®3000 UV/Visible Spectrophotometer (Pharmacia Biotech)) was programmed to measure the purity of the samples as a proportion of the signal at 260 nm and 280 nm. This proportion had to be ≥1.7 otherwise the sample was not pure enough. The cuvette (Präzisions-küvetten aus quarzglas SUPRASIL® (Hellma), light path: 10 mm, centre: 15 mm) was washed thoroughly with ultra pure water and the outside was dried with a piece of paper towel. 100 µl ultra pure water was added to the cuvette as a blank sample. The purity and concentration of the blank sample was measured and the cuvette was washed and dried. 100 µl of the first diluted sample was added to the cuvette and the purity and concentration of the sample was measured. The cuvette was then washed and dried. This procedure was repeated for all the samples and the resulting concentrations and purities were printed out.

2.4.6 Preparation and running of an agarose gel

We ran agarose gels to confirm that there was recombinant DNA in the samples before we started the actual experiments.

100 ml TBE buffer (10x) (see section 2.1.1 Preparation of TBE buffer (10x)) was diluted in 900 ml ultra pure water to make 1 L TBE buffer (1x). 1 g agarose (Agarose type I-A: low EEO (Sigma))

was added to the buffer and the flask was thoroughly shaken. The mixture was heated in a microwave oven until all the agarose was dissolved and the solution made pulses of foam. 15 µl Ethidium bromide (Sigma) was then added carefully. Gel Chamber DNA: Horizon®58 (Gibco BRL) and matching equipment were used to pour and run gels. The gel was run using TBE buffer (1x). The samples were prepared by adding 2 µl loading buffer (Gel loading buffer (Sigma)) to 10 µl of each DNA sample. 10 µl of each sample and 5 µl of the standard marker (Hyperladder 1 quantitative (Bioline)) were loaded onto the gel. The running parameters were 80 V for 30 minutes. The gel was photographed by using the ultraviolet transilluminator (Multilmage™ Light Cabinet (Alpha Innotech Corporation), software: Chemilmager™5500 Version 3.04C).

2.5 Transfection

2.5.1 Transfection of HEK 293T cells

HEK 293T cells were used to produce proteins from the different plasmid constructs. The day before the transfection, a confluent 75 cm^2 flask of HEK 293T cells was trypsinized (see section 2.3.3 Passaging HEK 293T) and the cells were seeded at 40-50% confluence in a 6-well plate (Nunc). The cells would then be between 50% and 90% confluent the next day. On the day of transfection the reaction mixtures were prepared separately for each well. In a 1.5 ml eppendorf tube 250 µl Optimem (OptiMEM®1 (GIBCO)) and 3 µg plasmid DNA were mixed gently by vortexing. In a second 1.5 ml eppendorf tube 250 µl Optimem and 10 µl Lipofectamine (Gibco) were mixed gently by vortexing. It was important that the Lipofectamine reagent was added directly in the Optimem and avoided contact with the eppendorf tube. After 5 minutes of incubation the two mixtures were combined and gently vortexed. The mixture was incubated at room temperature for 25 minutes and another 500 µl Optimem was added. 2 ml Optimem was gently added to the cells and the 6-well plate was rocked to thoroughly wash the monolayer. The Optimem was removed by using a vacuum pump. This procedure was repeated twice. The transfection mixture was added to the well and the cells were incubated at 37°C for 5 hours. The transfection mixture was then replaced by complete medium equilibrated to 37°C and the cells were incubated for 24-48 hours at 37°C. After the incubation the cells were lysed (see section 2.7.1 Lysing cells) and the amount of

proteins were quantified (see section 2.7.2 Quantification of proteins using the Bio-Rad *DC* protein assay kit).

2.6 Homogenization of human tissues

2.6.1 Tissue samples

Cardiac tissue samples obtained from patients with ischemic heart disease (IHD) or dilated cardiomyopathy (DCM) or from healthy 'donor' hearts were snap frozen in liquid nitrogen before use. Specimens were collected at Harefield Hospital by a collaborator, Dr. Najma Latif, in accordance with ethical procedures. Myocardial tissue was excised distal from the site of any myocardial infarcts from the diseased group of patients. Ventricular myocardial specimens were obtained by endomyocardial biopsy immediately prior to transplantation from the control donor hearts.

Biopsy specimens from healthy or diseased bowels were also snap-frozen in liquid nitrogen before use. They were collected by a collaborator, Professor Tom MacDonald, in accordance with ethical rules.

2.6.2 Homogenization of gut tissues

The Sample Grinding Kit from Amersham Biosciences was used. Ten 1.5 ml microcentrifuge grinding tubes, each containing a small quantity of abrasive grinding resin suspended in water, were centrifuged briefly at 14000 rpm to pellet the grinding resin. A pipette was used to remove as much of the liquid as possible from the grinding resin pellets. 100 µl complete lysis buffer (see section 2.1.2 Preparation of lysis buffer) was added to the grinding tubes and the tubes were vortexed to resuspend the grinding resin. The tissue samples were added to the tubes and pestles were used to thoroughly grind the samples. The tubes were centrifuged for 10 minutes at 14000 rpm to remove resin and cellular debris. The supernatants were carefully transferred to marked 1.5 eppendorf tubes and stored at -80^{o}C.

2.6.3 Homogenization of heart tissues

Snap frozen samples of human heart tissues were withdrawn from the -80°C freezer and put on ice immediately. An empty eppendorf tube was weighted on a fine-balance (Sartorius). The eppendorf tubes with the tissues were weighted and the weight of the tissues was calculated by subtracting the weight of the empty tube. From prior experiment we estimated that 0.3 g of tissue should be lysed using 1 ml lysis buffer (see section 2.1.2 Preparation of lysis buffer). The tissues were transferred to 5 ml homogenizing tubes (Polystyrene Round-Bottom tube, 5 ml (BD Falcon™)), an appropriate amount of lysis buffer was added and the tissues were homogenized (Ultra-turrax T25 (Janke&Kunkel IKA-Labortechnik), speed 13000-20000) on ice 2x30 seconds. When all the tissues had been homogenized the samples were transferred back into 1.5 ml marked eppendorf tubes. The tubes were centrifuged at 1400 rpm for 20 minutes at 4°C. The supernatants were separated from the pellets and the samples were stored at -80°C.

2.7 Preparation of cytosolic lysates and protein quantification

2.7.1 Lysing cells

10 ml of complete lysis buffer (see section 2.1.2 Preparation of lysis buffer) was prepared and put on ice. The medium was gently removed from the cells using a vacuum pump. An appropriate amount of ice cold sterile PBS (Phosphate buffered saline (OXOID)) was gently added to the cells and the flask/well was rocked to cover the monolayer (10 ml for a 75 cm^2 flask, 5 ml for a 25 cm^2 flask and 2 ml for a 9.6 cm^2 well respectively). The PBS was removed using a vacuum pump. This procedure was repeated twice. The desired amount of complete lysis buffer (150 µl for $14x10^6$ cells, 130 µl for a confluent 75 cm^2 flask, 130 µl for a confluent 25 cm^2 flask and 50 µl for a confluent 9.6 cm^2 well) was added to the cell monolayer and the flask/well was incubated on ice for 5 minutes. The cells were gathered using a cell scraper (VWR), and the cell suspension was transferred to a labeled 1.5 ml eppendorf tube. The tube was incubated on ice for 15 minutes and then spun down at 14000 rpm for 20 minutes at 4°C. The supernatant was removed from the pellet and placed in a labeled 1.5 ml eppendorf tube. Both the supernatant and the pellet were stored at -80°C.

2.7.2 Quantification of proteins using the Bio-Rad DC protein assay kit

The samples were diluted with complete lysis buffer (see section 2.1.2 Preparation of lysis buffer) so that their concentrations were within the range of the standard curve. Often several dilutions had to be made to make sure that at least one would fall within the desired concentration range. BSA solutions of 0.0 mg/ml, 0.2 mg/ml, 0.4 mg/ml, 0.6 mg/ml, 0.8 mg/ml, 1.0 mg/ml, 1.2 mg/ml and 1.4 mg/ml were made from a stock solution of 2 mg/ml BSA (Albumin from bovine serum, minimum 80% HPLC (Sigma)) and incomplete lysis buffer (see section 2.1.2 Preparation of lysis buffer). In a 96-well plate (F96 Maxisorp nunc-immuno plate (Nunc)), 5 µl of each sample and BSA dilutions was introduced in duplicate. 20 µl Reagent S (Bio-Rad *DC* Protein Assay Reagent S (BioRad)) was mixed with 1 ml Reagent A (Bio-Rad *DC* Protein Assay Reagent A (BioRad)) to give Solution A'. 25 µl Solution A' was added to the 5 µl of sample/BSA solution. 200 µl Reagent B (Bio-Rad *DC* Protein Assay Reagent B (BioRad)) was then added to each well and the reaction was allowed to proceed for 5-15 minutes until the color of the liquid in the wells had turned blue. The plate was centrifuged at 1200 rpm for 30 seconds (Universal 16R (Meadowrose scientific LTD)). The plate was inserted into the plate reader (DYNEX technologies MRX, software: Revelation version 3.2), which analyzed the content and calculated the concentrations of the different samples.

As a precaution the calculations were double-checked using Microsoft Excel. By using the values given by the plate reader a standard curve was made for the concentrations of the different BSA solutions (see 'Results' section 3.1.1). In addition to make the standard curve the computer provided the equation of the graph and the R^2 value. R^2 corresponds to the coefficient of determination. This value gives the proportion of the variance of one variable that is predictable from the other variable. R^2 is the ratio of the explained variation to the total variation. It ranges from $0 < R^2 < 1$, and denotes the strength of the linear association between two variables. The higher the value of R^2, the better is the linear relationship between x and y (63). The equation made from the standard curve could be used to calculate the concentrations of the different samples. The general term for this type of equation is $y = ax + b$, where y and x are variables and a and b are

constants. In this particular equation y corresponded to the ABS value, and x corresponded to the concentration:

$$y = ax + b$$

$$ABS = a\ concentration + b$$

$$concentration = (ABS - b)/a$$

This equation was used to calculate the concentration of the different samples by using the average of the ABS values given by the plate reader. In the Bio-Rad *DC* protein assay the different samples were diluted so that their concentrations would fall within the area of the standard curve. To get the final concentration this dilution factor had to be taken into consideration. The final concentration was obtained by multiplying the concentration by the dilution factor. If 10 µg of proteins was to be used in the western blots, the volume of sample needed was calculated by dividing 10 µg by the values obtained for the final concentrations. Finally the volume of lysis buffer needed to get a total volume of 6 µl for the reaction with the HAUbVME probe was calculated.

2.8 Identification of DUBs using a thiol-reactive probe

2.8.1 Identification of DUBs using the HAUbVME probe

As a negative control for the probe reaction a sample was always run in duplicate. One of these samples received the HAUbVME probe (provided by Ph.D. Huib Ovaa, Division of cellular biochemistry, Netherlands Cancer Institute), while the other did not.

For each sample a volume corresponding to 10 µg proteins or a maximum of 6 µl sample was used. The volume was made up to 6 µl by adding lysis buffer (see section 2.1.2 Preparation of lysis buffer). Two master mixes were made, one containing the HAUbVME probe and one without it. For each sample the master mixes contained 1.5 µl 30 mM DTT (DL-Dithiothreitol (Sigma)) and 6.5 µl reaction buffer (see section 2.1.3 Preparation of reaction buffer). The second master mix also contained 0.25 µl HAUbVME probe per sample. The samples that were not to receive the probe was added 8 µl of the first master mix, while the samples that were going to receive the probe were

added 8.25 µl of the second master mix. After thorough mixing the samples were incubated at 37°C for 1 hour.

Previous studies using the probe-binding technology have identified DUBs at a sequence level using proteomic approaches (15,19). The identity of particular DUBs in our experiment was inferred by reference to these earlier studies.

2.9 Protein analysis by western blotting

2.9.1 SDS Page Electrophoresis

A master mix of DTT (DL-Dithiothreitol (Sigma)) and NuPAGE Sample buffer (NuPAGE™ LDS Sample Buffer (4x) (Invitrogen)) was prepared. 2.97 µl 1 M DTT per 20 µl sample and 6.67 µl NuPAGE Sample buffer per 20 µl sample were used. The samples were mixed well by pipetting. A hole was made in each tube to avoid an outburst during the heat denaturising. Each sample was incubated at 98°C for 5 minutes. The samples were then centrifuged briefly at 14000 rpm to retrieve condensation.

Samples were run using the NuPAGE™ Gel System from Invitrogen. Samples were loaded on NuPAGE™ 4-12% Bis-Tris electrophoresis gels with 12 wells of 1.0 mm. Gels were run using 800 ml running buffer (NuPAGE™ MOPS SDS Running Buffer (20x) (Invitrogen)) at 200 V for 1 hour. 6 µl standard marker (SeeBlue Plus2® Prestained standard (1x) (Invitrogen)) and 20 µl of the samples were loaded.

2.9.2 Western blotting

Transfer was performed using the NuPAGE™ System (Invitrogen). 800 ml complete transfer buffer was made consisting of 679 ml ultrapure water, 40 ml transfer buffer (NuPAGE™ Transfer Buffer (20x) (Invitrogen)), 1 ml antioxidant (NuPAGE™ Antioxidant (Invitrogen)) and 80 ml methanol (Methanol Microscopy (BDH)). A PVDF membrane (Immobilon-P Transfer membrane, filter type: PVDF, pore size: 0.45 µm (Millipore)) was wetted for 30 seconds in methanol (Methanol Microscopy (BDH)) and then washed quickly in the transfer buffer before use. Transfer was completed by assembling the gel, membrane, filter paper (Chromatography paper, 3mm, CHR,

58x68 cm (Whatman®) (soaked in transfer buffer) and blotting pads (soaked in transfer buffer) as shown below (Figure 2.9.1).

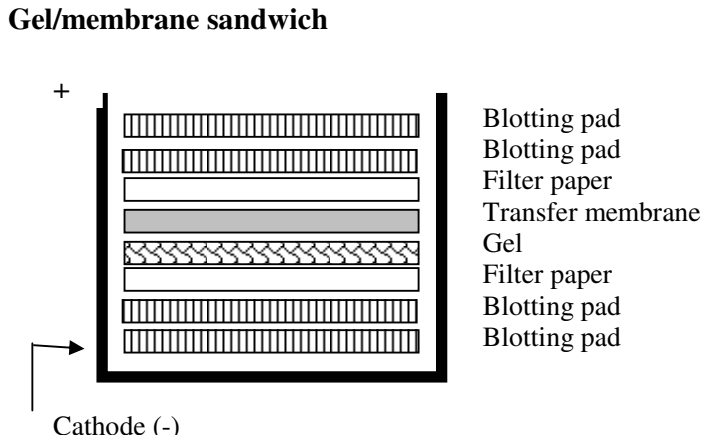

Figure 2.9.1: The western blotting sandwich.

The sandwich was placed in the western blot box and filled with transfer buffer. Tap water was added to the outermost part of the box to keep the system cold during the transfer. The parameters were 25 V for 1.5 hours.

2.9.3 Blocking and protein detection

Membranes, which were to receive the ubiquitin antibody, had to be autoclaved before blocking. The membrane was wetted in PBS (Phosphate buffered saline (OXOID)) and placed between six sheets of filter paper soaked in PBS. The sandwich was covered with foil and autoclaved (Napco® Model 8000-DSE autoclave, parameters: 121^{o}C for 11 minutes).

The membrane, containing transferred proteins, was placed with the protein side upwards in a box containing 50 ml block solution consisting of 5% Marvel milk (Marvel dried skimmed milk, 1% fat) in a 0.1% PBS-Tween solution (500 µl Tween®20 Sigma Ultra (Sigma) in 500 ml Phosphate buffered saline (OXOID)). The box was left on a shaker for 1 hour at room temperature or over night at 4^{o}C. After the blocking step the membrane was rinsed thoroughly in 0.1% PBS-Tween to remove the excess of milk. 20 ml of the primary antibody solution was made up and poured over the membrane (Table 2.9.1).

Table 2.9.1: Primary antibodies.

Primary antibody	Dilution factor	Diluted in
Anti-HA High Affinity (Roche)	1/1000	0.1% PBS-Tween
DUB-2 (rabbit polyclonal no. 305) (BD)	1/1000	0.1% PBS-Tween containing 5% Marvel milk
Mouse anti-Ubiquitin (0.8 mg/ml) (Zymed)	1/1000	0.1% PBS-Tween
Monoclonal Anti-α-Tubulin Antibody produced in mouse (Sigma)	1/5000	0.1% PBS-Tween
Actin (C-2) (Santa Cruz Biotechnology)	1/1000	0.1% PBS-Tween
Mouse anti-Glyceraldehyde-3-phosphate dehydrogenase monoclonal antibody (GAPDH) (Chemicon)	1/10,000	0.1% PBS-Tween
Rabbit polyclonal to HAUSP (ab4080) (Abcam)	1/1000	0.1% PBS-Tween
Cytokeratin19 clone BA17 (DAKO)	1/10,000	0.1% PBS-Tween added 0.1% BSA

The box was left on a shaker for 1 hour at room temperature or over night at 4^{o}C. While still on the shaker the membrane was rinsed one time with 0.1% PBS-Tween for 15 minutes and then three times for 5 minutes each. 15 ml of the secondary antibody solution was then prepared and incubated with the membrane (all secondary antibodies were diluted in 0.1% PBS-Tween) (Table 2.9.2).

Table 2.9.2: Secondary antibodies.

Primary antibody	Secondary antibody	Dilution factor
α-HA	Goat anti rat IgG Peroxidase conjugated (Chemicon)	1/5000
α-DUB2	Polyclonal Goat Anti-Rabbit Immunoglobulins/HRP (DakoCytomation)	1/10,000
α-Ubiquitin	Polyclonal Goat Anti-Mouse Immunoglobulins/HRP (DakoCytomation)	1/10,000
α-Tubulin	Polyclonal Goat Anti-Mouse Immunoglobulins/HRP (DakoCytomation)	1/10,000
α-Actin	Polyclonal Goat Anti-Mouse Immunoglobulins/HRP (DakoCytomation)	1/10,000
α-GAPDH	Polyclonal Goat Anti-Mouse Immunoglobulins/HRP (DakoCytomation)	1/10,000
α-HAUSP	Polyclonal Goat Anti-Rabbit Immunoglobulins/HRP (DakoCytomation)	1/10,000
α-Cytokeratin19	Polyclonal Goat Anti-Mouse Immunoglobulins/HRP (DakoCytomation)	1/10,000

The secondary antibody was left on for 40 minutes and then the membrane was washed using 0.1% PBS-Tween (1x15 minutes and 3x5 minutes). 8 ml Enhanced chemiluminesent substrate was prepared according to manufacturers instructions (Western Lightning™Chemiluminescence reagents (PerkinElmer)). The membrane was dried quickly between two sheets of filter paper and incubated with substrate for 2 minutes. The membrane was dried quickly between two sheets of filter paper, wrapped in saran film (Saran barrier food wrap (Dow)) and placed in a cassette tight to light. In the dark room a photographic film (KODAK®BioMax Light Film 18x24 cm Light-1 (Sigma-Aldrich)) was placed above the membrane for an appropriate amount of time and the film was developed using the developer machine (Compact X4 Automatic X-ray Film Processor (X-ograph Imaging Systems)).

When it was difficult to analyze the result with the naked eye, bands were quantified using densitometry (Software: Chemilmager™5500 Version 3.04C). The densitometry data was analyzed using Microsoft® Office Exel 2003.

2.9.4 Comparison of protein loading by Coomassie blue staining

The blot from the western blotting procedure (see section 2.9 Protein analysis by western blotting) was incubated with Coomassie Brilliant Blue R-250 Staining Solution (BioRad) for about 30 minutes on a shaker. The membrane was then washed using destaining solution (see section 2.1.4 Preparation of Coomassie blue destaining solution) for about 15 minutes. The destaining solution was poured away and replaced by the same amount of new destaining solution. The box was left on the shaker for another 15 minutes. This procedure was continued until almost all the background staining on the blot was washed away. When the washing procedure was finished, the blot was placed between two layers of filter paper to dry.

2.10 Induction of hypoxic effects in HUVEC

2.10.1 Growing cells in hypoxia to study hypoxic effects

The Invivo$_2$ 1000 hypoxic workstation (Ruskinn) provides a suitable environment for cell culture experiments in which the oxygen concentration needs to be tightly controlled for short or long periods. The workstation has two separate chambers, one of which enables the investigator to carry out procedures in anoxia, and the other in a controlled oxygen environment (we used 1% oxygen in our experiments). The equipment has two gloved access ports for each chamber, thereby allowing sample handling. Items can be placed in either chamber via an interlock, or via a small mailbox on the side (Figure 2.10.1).

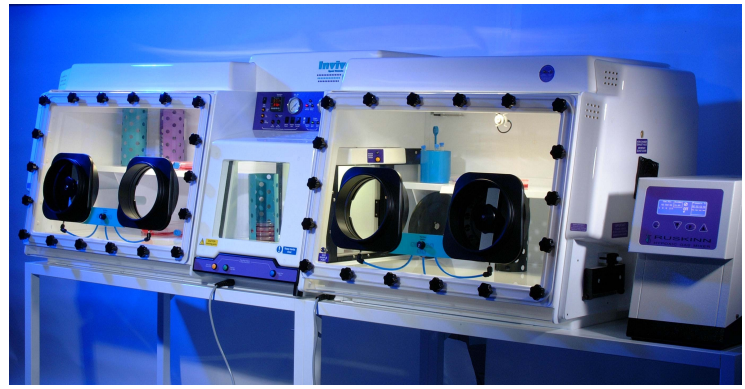

Figure 2.10.1: The Invivo$_2$ 1000 hypoxic workstation. Picture obtained from Miguel A. Esteban.

Cultures of HUVEC were grown until they were about 90% confluent. The cells were then placed in the hypoxic chamber for the desired amount of time. If we wanted to induce translocation of NF-κB into the nucleus, the cells were given 20 ng/ml IL-1 (Human IL-1 (Peprotech)) for the final 30 minutes of the incubation time. Addition of IL-1 and fixation of the cells using paraformaldehyde (see section 2.11.1 Staining HUVEC for p65) was done within the hypoxic chamber.

2.11 Immunofluorescence of cultured cells grown on glass coverslips

2.11.1 Staining HUVEC for p65

The medium was removed from HUVEC grown on glass coverslips (Cover Glass 22x22 mm (VWR)) placed in small dishes (Cell culture dish 35 mm x 10 mm style (Corning)). The monolayer was rinsed using 2 ml PBS (Phosphate buffered saline (OXOID)) before the cells were fixed using 4% paraformaldehyde (Sigma) for 10 minutes. The monolayer was then rinsed three times with 2 ml PBS. The cells were permeabilized in 0.1% Triton X-100/PBS (400 µl Triton X-100 (Sigma) in 400 ml Phosphate buffered saline (OXOID)) for 2x4 minutes. The permeabilization was followed by three successive rinses using 2 ml PBS/0.5% bovine serum albumin (BSA) (5 ml Albumin solution, from bovine serum 30% (Sigma) in 295 ml Phosphate buffered saline (OXOID)). The coverslips were then incubated with blocking buffer (PBS/0.5% BSA) for 30 minutes. The cells

were incubated with the primary antibody (NF-κB p65 (C-20) (Santa Cruz Biotechnology) (Dilution: 1/200)) in blocking buffer for one hour. The coverslips were rinsed two times with 2 ml blocking buffer followed by 3x10 minutes washes with blocking buffer. The cells were then incubated with the secondary antibody (Alexa Fluor® 568 goat anti-rabbit IgG (Molecular Probes) (Dilution: 1/300)) in blocking buffer for 45 minutes. The coverslips were rinsed two times with blocking buffer and washed 3x10 minutes with blocking buffer. The cells were then rinsed in PBS, dipped in water and mounted (Aqua Poly/Mount (Polysciences.Inc)).

The cells were photographed and analyzed using confocal microscopy (Axiovert 200M Confocal support (ZEISS), Software: LSM 510). Numeric data was visualized using the GraphPad Prism version 4.00 software.

3. RESULTS

3.1 Expression patterns of novel 50 kD and 60 kD DUBs identified in T-cells

3.1.1 Experiments to reveal the identity of DUBs found in proliferating CD4+ T-cells

My supervisor and co-workers had been working with the ubiquitin-derived thiol-reactive probe HAUbVME before I joined them in the Intracellular Signaling Laboratory, Imperial College. They had discovered two proteins, which were present in proliferating CD4+ T-cells, but not in non-proliferating CD4+ T-cells. They had a theory that these molecules regulate the development of T-lymphocytes or their subsequent responses to antigen.

The size of the UBP DUB-2 is approximately 62 kD (16,20,21). Its expression is induced by IL-2 and is restricted to T-cells. The size and expression pattern of DUB-2 corresponded well to the proteins found at 60 kD and 70 kD combined with probe in proliferating CD4+ T-cells, as the size of these proteins without the probe would be about 50 kD and 60 kD respectively. We therefore decided to investigate if either of these proteins corresponded to DUB-2.

DUB-2 expression plasmids

We received three different plasmids of which two encoded active DUB-2 and one encoded an inactive form of DUB-2 where a cysteine in the catalytic site had been replaced by a serine unit (DUB-2 cs). We amplified the amount of DNA by transforming the different plasmids into *Escherichia coli*. To separate the DNA from the bacteria we did midipreps of one of the DUB-2 cDNAs and the DUB-2 cs cDNA and miniprep of the other DUB-2 cDNA. To determine the purity and concentration of the obtained DNA solutions, the absorbance of UV light was measured in a spectrophotometer. All our DNA solutions had ratios above 1.7, which meant they were pure enough to be used in experiments. The purity and concentrations were as follows:

Table 3.1.1: Data from the spectrophotometer

Sample	260 nm	280 nm	Ratio	Concentration
DUB-2 #1	0.218	0.117	1.86	10.90
DUB-2 #2	0.280	0.235	1.80	4.95
DUB-2 cs	0.596	0.337	1.770	29.80

We wanted to confirm that there was recombinant DNA in the samples before we started the actual experiment. To do this we ran an agarose gel. The DNA was visualized by addition of ethidium bromide in the gel (Figure 3.1.1).

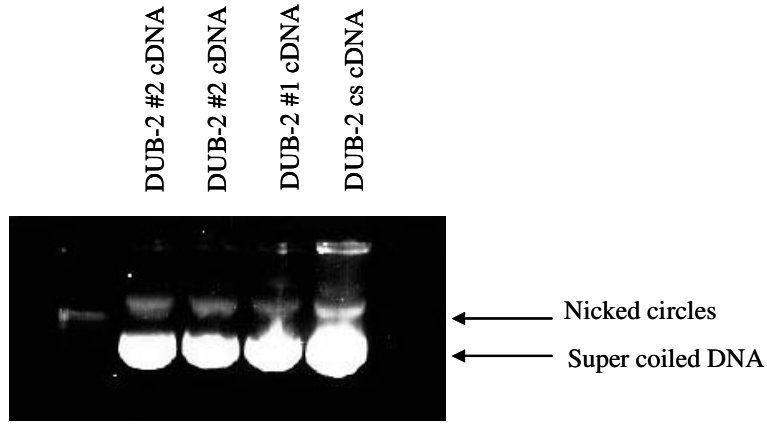

Figure 3.1.1: Agarose gel showing the DUB-2 and DUB-2 cs plasmids.

The gel confirmed that there was intact DNA in all the samples. The picture shows at least two different topological forms of the DNA in each lane. The brightest bands correspond to super coiled DNA whereas the smaller bands above correspond to nicked circles.

Production of DUB-2 protein in HEK 293T cells

The next step was to produce large amounts of the translation products of the three different plasmids. This was done by transfecting the plasmids into HEK 293T cells. We used the lipofectamine procedure to introduce the DNA into the cells.

When the transfection was completed the cells were lysed and the amount of protein was quantified to normalize samples. From the data obtained from the plate-reader we calculated the amount of lysate necessary to get 10 µg of proteins from each sample. We did this by making a

standard curve from the mean absorbance (ABS) values given for the different concentrations of the BSA solution (see appendix for the printout from the plate-reader) (Figure 3.1.2).

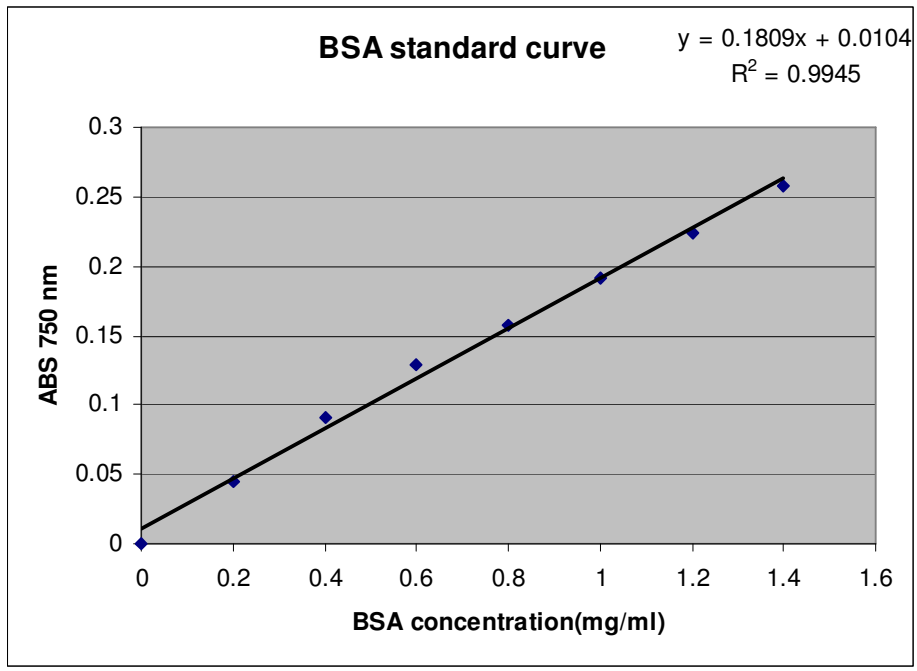

Figure 3.1.2: BSA standard curve showing the absorbance values as a function of the different BSA concentrations. The equation and the R^2 value are given in the upper right corner.

From the equation obtained from the standard curve we calculated the concentrations of the different samples by using the method given in 'Materials and methods' section 2.7.2.

Probing lysates using HAUbVME

After the quantification we ran the probe-binding reaction and a western blot for HA to detect probe-DUB conjugates (Figure 3.1.3)

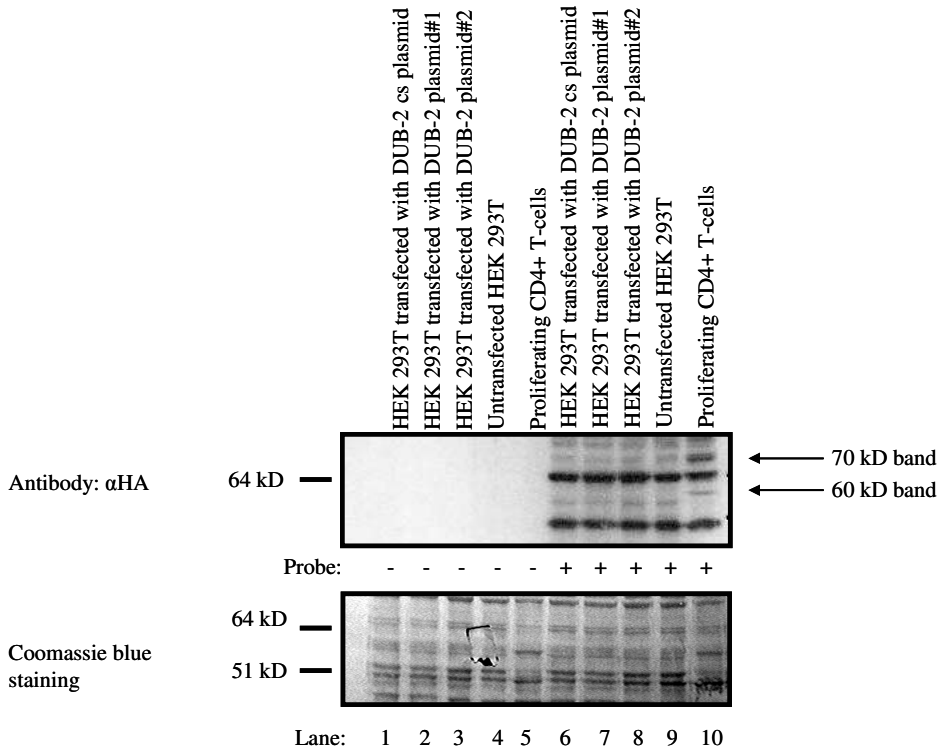

Figure 3.1.3: Western blot for HA: Comparison between DUB-2 and 70 kD and 60 kD DUBs found in T-cells.

Probing for DUBs revealed both the 50 kD and the 60 kD protein in the T-cell lysate. Since the proteins are combined with probe they appear at about 60 kD and 70 kD respectively (as indicated; (lane 10)). The 60 kD band is clearly not present in HEK 293T cells expressing DUB-2 (lanes 7, 8). However, there is a faint band in all HEK 293T lysates at the same size as the 70 kD band (lanes 6, 7, 8, 9). The lysates from untransfected HEK 293T cells were included as a control. This lysate shows the normal pattern of active DUBs in these cells.

The active site in the translation product from the DUB-2 cs DNA is mutated, so this DUB should not be able to bind the probe. This means that there should be no bands corresponding to DUB-2 cs in the lysate from cells transfected with DUB-2 cs (lane 6). The active site of DUB-2 however, should be able to bind covalently to the probe and we might expect a band corresponding to DUB-2 in the lysates from the cells transfected with the DUB-2 DNA (lanes 7, 8). There are no

obvious differences, however, between cells expressing active or inactive DUB-2 (compare lanes 6, 7, 8).

There are no bands in the lanes with the samples that did not receive the probe, which means that there is no unspecific binding of the antibodies. The Coomassie blue staining shows that the loading was equal for all the samples.

We ran a second western blot in parallel with the first one. This membrane was incubated with an anti DUB-2 antibody. We ran two sets of samples, of which one set received the HAUbVME probe while the other did not (Figure 3.1.4).

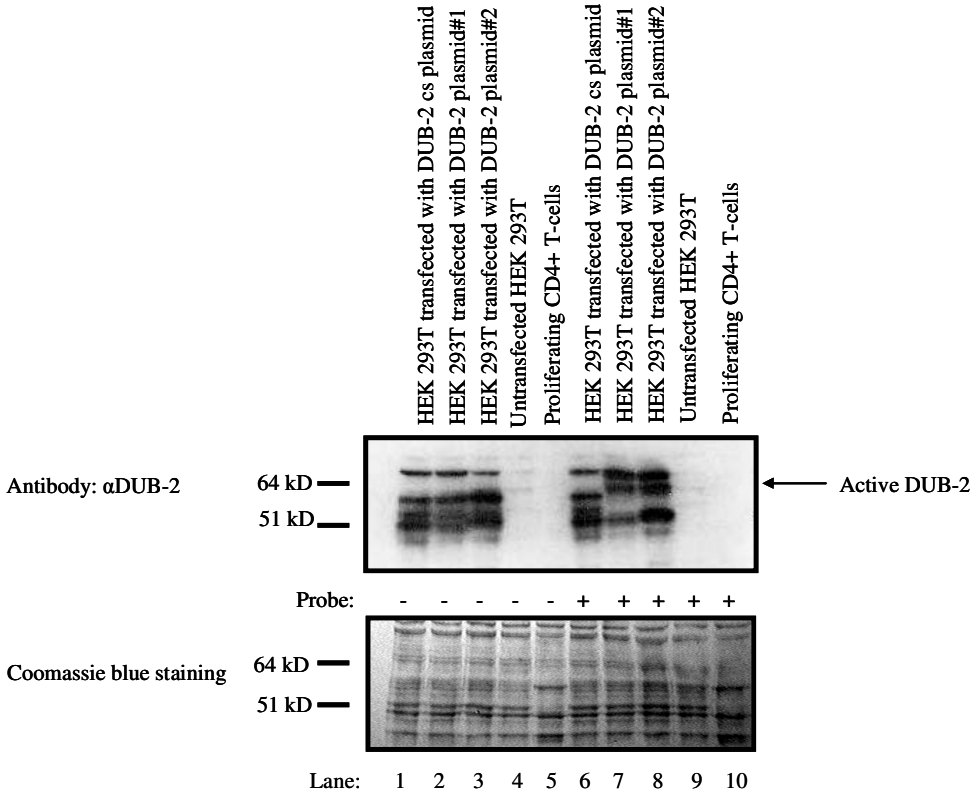

Figure 3.1.4: Western blot for DUB-2: Identification of DUBs in CD4+ T-cells.

There are no signals in the lanes with the proliferating CD4+ T-cells, neither for the sample that did receive the HAUbVME probe (lane 10) nor for the one that was not combined with the probe (lane 5). This suggests strongly that the 60 kD and 70 kD bands detected with the probe in CD4+ T-cells do not correspond to DUB-2.

The picture shows the presence of many different forms of both DUB-2 and DUB-2 cs in the lysates from the transfected HEK 293T cells. The signaling pattern for DUB-2 cs is the same both with and without the HAUbVME probe (compare lanes 1 and 6). For DUB-2 however, some of the forms are shifted to a higher level when combined to the probe (Active DUB-2), while other forms are not elevated by the presence of the HAUbVME probe (compare lanes 2 and 7, lanes 3 and 8). The higher forms in lanes 7 and 8 correspond to DUB-2-probe conjugates. It is possible that they were not detected using anti-HA antibodies because of a lower sensitivity of anti-HA antibodies compared to anti DUB-2 antibodies. Note that the Coomassie blue staining suggests that the amount of proteins loaded is equal for all the samples.

We concluded that neither of the two novel DUBs found in proliferating CD4+ T-cells was DUB-2.

3.1.2 Are the DUBs found in proliferating CD4+ T-cells also found in HUVEC?

The expression of DUB-2 is restricted to T-lymphocytes (3). However, since we confirmed that neither of the two DUBs found in proliferating CD4+ T-cells was DUB-2 we wanted to find out if these proteins are T-cell specific or if they appear in other proliferating cells as well.

To do this we compared proliferating and non-proliferating endothelial cell cultures. We grew cultures of proliferating and non-proliferating HUVEC using two different methods. In the first method (Method 1) cells were seeded at 90%, 50% and 30% confluence and the amount of FCS and ECGS was held constant during the entire growth period. The amounts were 20% and 10 µl/ml respectively. This method relied on the fact that proliferation is stopped by contact inhibition. In the second method (Method 2) cells, seeded at 90%, 50% and 30% respectively, were first given 20% FCS and just 2.5 µl ECGS for 16 hours. The cells plated out at 90% confluence were then given a new medium containing 2.5% FCS and 2.5 µl/ml ECGS. The idea was that the reduced amount of FCS and ECGS would stop the growth of these cells. The cells seeded at 50% and 30% confluence were given a medium containing 2.5% FCS and 10 µl/ml ECGS. The increased amount of ECGS given to these cells, as compared to the ones seeded at 90% confluence, ensured continued proliferation.

Probing lysates using HAUbVME

When the cells had reached the desired confluence they were lysed and the protein load was quantified. Identification of DUBs was performed using the HAUbVME probe followed by SDS Page electrophoresis and western blot for HA to detect probe-DUB conjugates. The loading was controlled by Coomassie blue staining.

As a control for the probe-binding reaction we included lysates from untransfected HEK 293T cells made in the DUB-2 experiment. One of these samples received the probe while the other did not. Lysate from proliferating CD4+ T-cells was already prepared and was included as a positive control for the two bands of current interest, which should appear at about 60 kD and 70 kD combined with probe respectively (Figure 3.1.5).

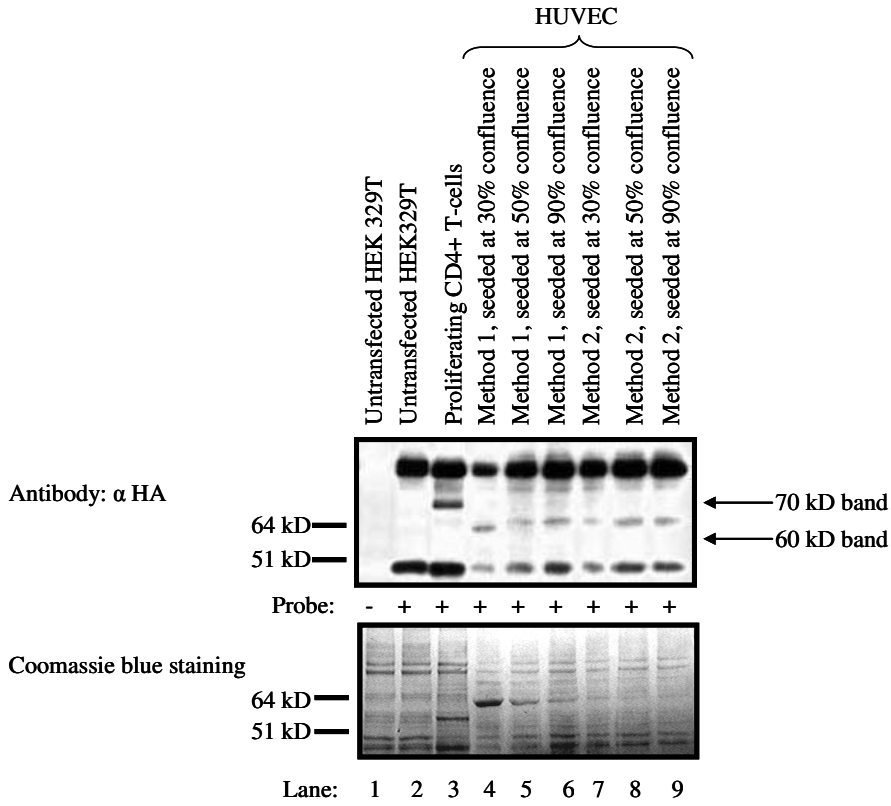

Figure 3.1.5: Western blot for HA: Comparison of the composition of DUBs in CD4+ T-cells and HUVEC cells.

The 70 kD band, which corresponds to one of the proteins of interest, is quite dense in the lysate from the proliferating CD4+ T-cells (lane 3). At first glance it might look like there is a really faint band at this position in the HUVEC (Method 1) samples seeded at 50% and 90% confluence (lanes 5, 6), but when we look closer it looks more like background disturbance than actual bands. There are no bands at this position in the other HUVEC samples.

The band of interest at 60 kD in the T-cell lysate is barely visible (lane 3). A close look at the film confirms that there is a faint band present at about 60 kD in the lysate from the proliferating CD4+ T-cells that is not apparent in HUVEC samples. To substantiate this finding we did a densitometry analysis (Figure 3.1.6).

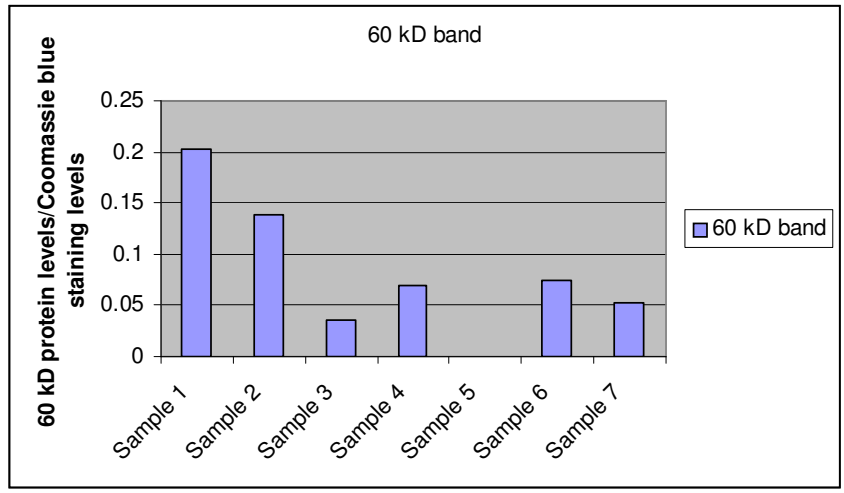

Figure 3.1.6: Densitometry analysis for the band at 60 kD. Sample 1 = Proliferating CD4+ T-cells, Sample 2 = HUVEC 'Method 1, seeded at 30% confluence', Sample 3 = HUVEC 'Method 1, seeded at 50% confluence', Sample 4 = HUVEC 'Method 1, seeded at 90% confluence', Sample 5 = HUVEC 'Method 2, seeded at 30% confluence', Sample 6 = HUVEC 'Method 2, seeded at 50% confluence', Sample 7 = HUVEC 'Method 2, seeded at 90% confluence'.

The graph shows that there is a band at 60 kD CD4+ T-cells (sample 1, lane 3). The intensity of this band is low in the HUVEC samples, so we can conclude that the 60 kD DUB (50 kD without probe) is not present in HUVEC.

There is a band just underneath the 64 kD mark in the HUVEC (Method 1) sample seeded at 30% confluence (lane 4) which is not present in the other HUVEC samples. There are no bands

appearing in the lane with the sample that did not receive the HAUbVME probe, which means that there is no unspecific binding of the antibodies. The Coomassie blue staining shows that the loading is perhaps a bit lower for the HUVEC (Method 1) samples seeded at 30% and 50% confluence then for the other samples (lanes 4, 5).

We concluded that the 50 kD and 60 kD DUBs (corresponding to 60 kD and 70 kD probe-DUB conjugates) were not expressed in endothelial cells and were likely to be T-cell specific.

Identification of these DUBs at a sequence level would require their purification and analysis using proteomics methods. This particular approach was, however, beyond the remit of this project.

3.2 Identification of DUBs in nuclear lysates in different stages of the cell cycle

3.2.1 Background

We received samples from the Cancer Genetics Laboratory at Guy's hospital with the request of analyzing them for differences in DUBs and levels of ubiquitinated proteins. The samples consisted of nuclear lysates from cells in different stages of the cell cycle (Table 3.2.1).

Table 3.2.1: Details about the different samples.

Number	Label	Protein concentration (mg/ml)	Cell phase (%)		
			G1	S	G2/M
1	Async	3	56	20	21
2	G1	2	80	15	5
3	S-phase	0.6	27	41	27
4	S, HU	0.6	44	37	16
5	S, IR	0.6	37	39	21
6	S-Low-IR	0.8	38	37	22
7	M-Col	1.5	28	28	42

The cells had been synchronized and treated according to (35) and the nuclear extracts had been prepared according to (64). The cells in the 'Async' sample had not been synchronized. The G1 cells had been serum starved for 48 hours. The S-phase cells had been starved for 24 hours and then released into 20% FCS media for a further 24 hours. The 'S, HU' sample consisted of S-phase synchronized cells that had been treated with 3 mM hydroxyurea (HU). HU causes replication fork

stalling, leading to double strand breaks. The 'S, IR' cells were S-phase cells exposed to 10 Gray irradiation, which is a lethal does of irradiation that causes many DNA double strand breaks. The 'S-Low-IR' cells had been exposed to 1 Gray irradiation and given 6 hours to recover. This treatment also causes breaks in DNA, but the lower dose and longer recover time were designed to induce the G2/M checkpoint, which occurs between the G2-phase and the M-phase (although the cell phase (%) data shows that this may not have worked). The 'M-Col' sample consisted of M-phase cells treated with colcimin, a microtubule inhibitor.

Our collaborators had previous obtained some data which suggested that S-phase cells, and particularly S-phase cells treated with DNA damaging agents, produce ubiquitin conjugates trough the action of the E3 ubiquitin ligase BRCA1 (35). They contacted us to find out whether the profile of nuclear DUBs was altered within the cell cycle and specifically with DNA damaging treatments.

Quality control

As a quality control measure we decided to run a western blot for a cytoplasmic protein called GAPDH (Figure 3.2.1).

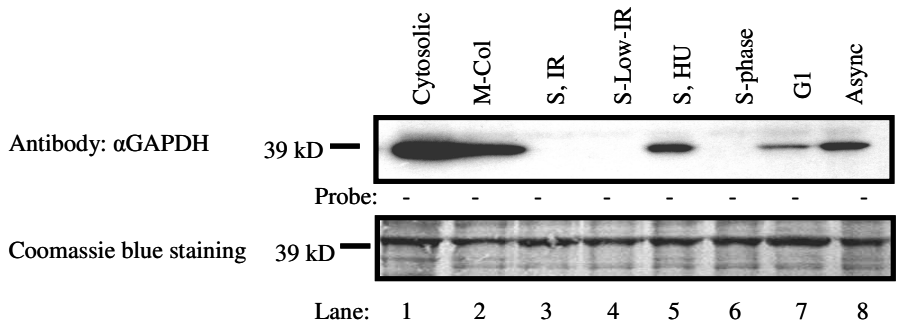

Figure 3.2.1: Western blot for GAPDH: Loading control.

GAPDH was present in several nuclear samples suggesting that these isolates may contain 'contaminating' cytosolic proteins. Therefore the results obtained from 'M-Col', 'S, HU', 'G1' and 'Async' samples should be interpreted with caution.

3.2.2 Levels of active DUBs

Probing lysates using HAUbVME

First we wanted to find out if there were any differences in the levels of active DUBs between the samples. Since we had never run the probe-binding reaction on nuclear lysates before, we included a sample containing cytosolic lysate as a positive control. Identification of DUBs was performed using the HAUbVME probe and western blotting for HA. The loading was controlled by Coomassie blue staining. Bands are discussed using arbitrary labels (w, x, y, z). Previous studies using the probe-binding technology have identified DUBs at a sequence level using proteomic approaches (15,19). The identity of particular DUBs in our experiments was inferred by reference to these earlier studies (Figure 3.2.2).

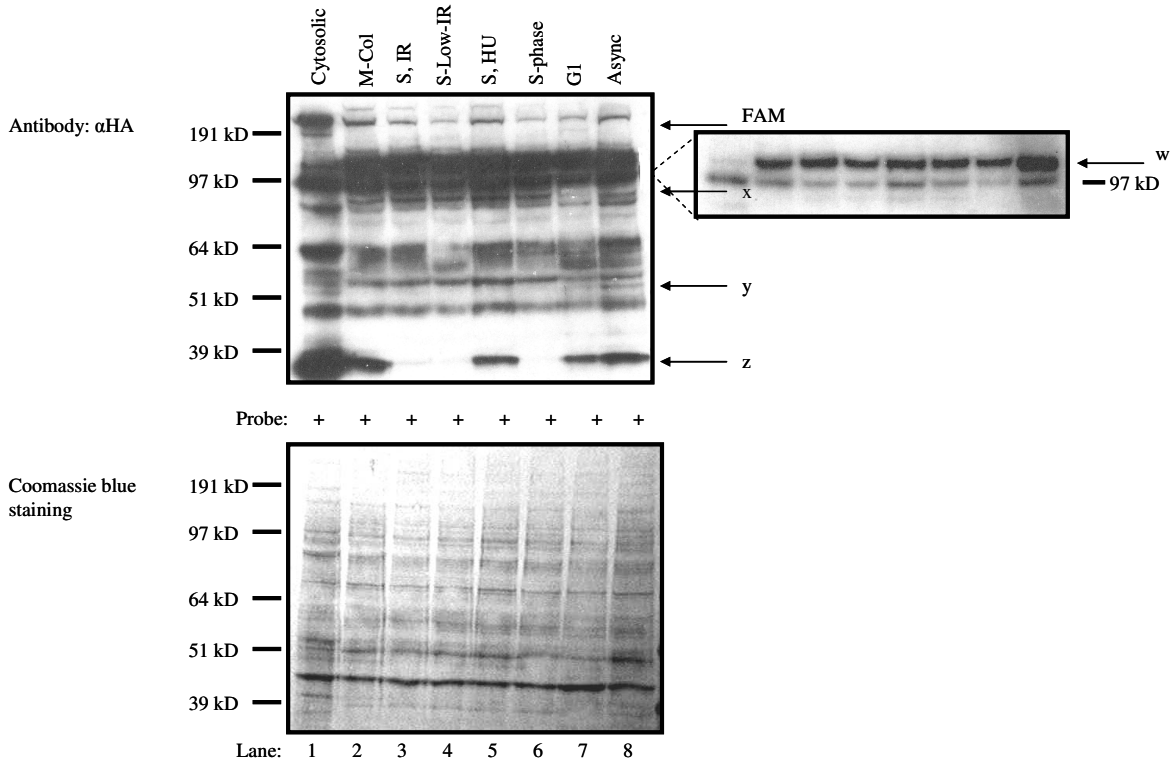

Figure 3.2.2: Western blot for HA: Levels of DUBs in different parts of the cell cycle after various treatments. Highlighted: Nuclear-specific DUB.

The band w is much denser in the nuclear samples than in the cytosolic sample. This indicates that a DUB of approximately 100 kD combined with probe is expressed specific in the nucleus.

Band x is also restricted to the nuclear lysates. FAM however, seems to be a cytosol-specific DUB. There is also a DUB of approximately 40 kD (51 kD with probe; band y), which is present in two of the nuclear samples (lanes 7, 8) as well as in the cytosolic lysate (lane 1), but not in the other samples. We feel that these results are valid despite the cytosolic contamination observed in some nuclear extracts.

We also observed decreased levels of a DUB (z) in three of the nuclear lysates (lanes 3, 4, 6). The intensity of this band is very high in the cytosolic sample compared to the nuclear lysates. We feel that this binding pattern may result from cytosol contamination of lanes 2, 5, 7, 8.

The Coomassie blue staining suggests that the loading is equal for all the samples. As a control for the probe-binding reaction we ran a second western blot in parallel with the first one. These samples did not receive the probe (Figure 3.2.3).

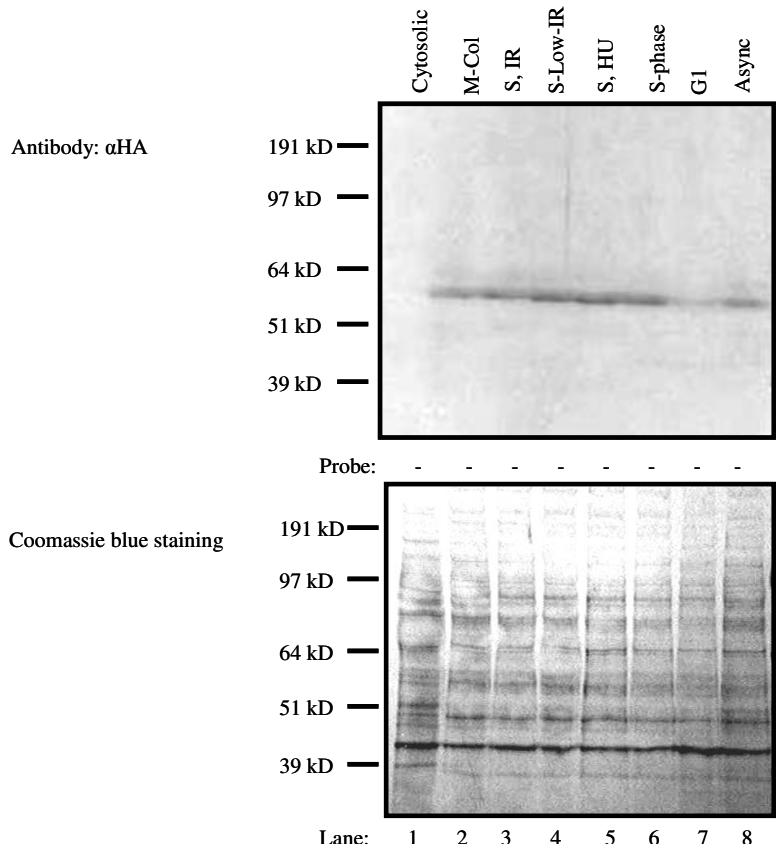

Figure 3.2.3: Western blot for HA: Negative control for the probe-binding reaction.

The picture shows some unspecific binding of one or both antibodies between 51 and 64 kD.

3.2.3 Levels of ubiquitinated proteins

Next we wanted to see if there were any differences in the level of ubiquitinated proteins between the samples. To do this we ran a western blot for Ub followed by Coomassie blue staining to control the loading. We used 20 µg of each sample (Figure 3.2.4).

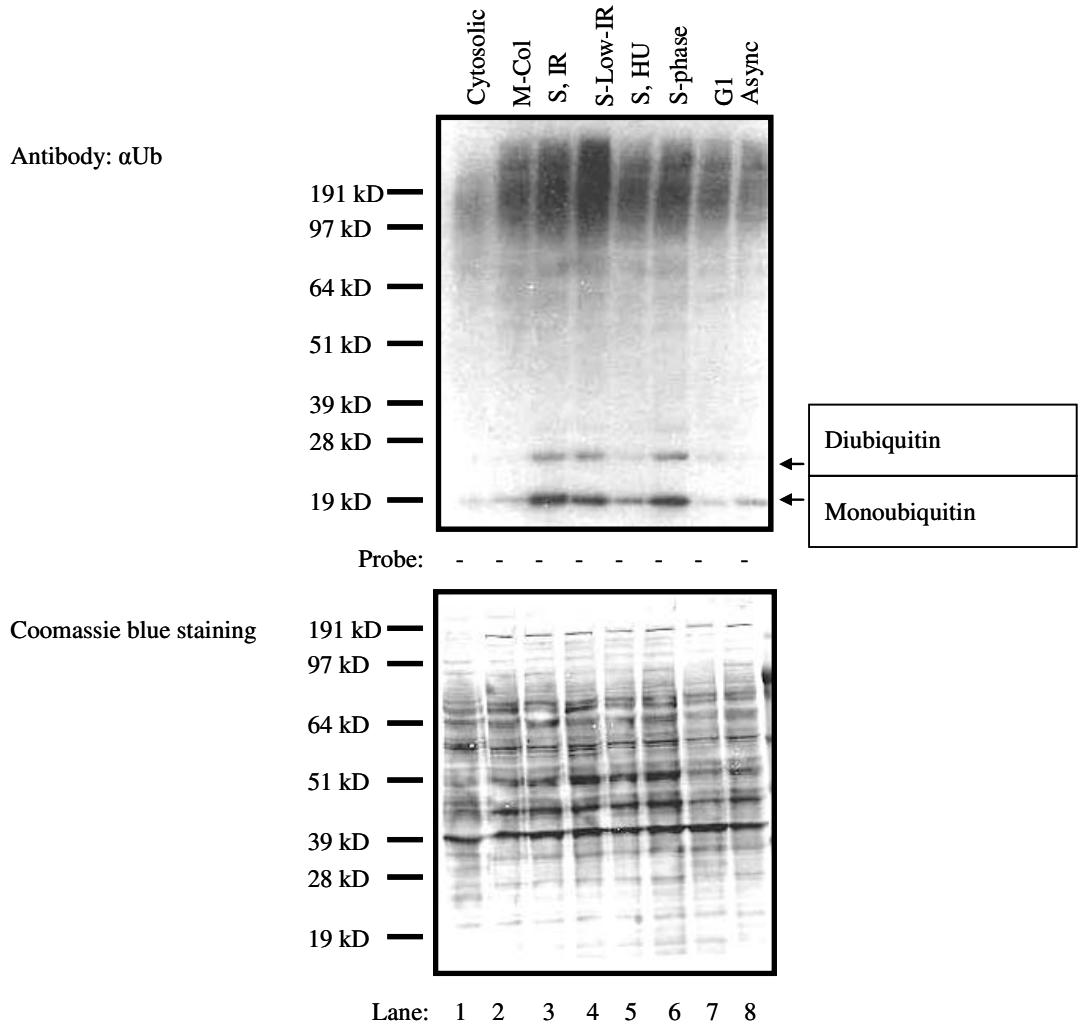

Figure 3.2.4: Western blot for Ub: The levels of ubiquitinated proteins in nuclear- and cytosolic extracts.

The level of ubiquitinated proteins is clearly higher for the nuclear lysates then for the cytosolic sample. It also seems like the level is a bit higher for the 'S-Low-IR' sample (lane 4) than for the

other nuclear samples. The levels of both mono- and diubiquitin vary between the samples. The three nuclear samples that showed low expression of the DUB below 39 kD in the HA blot show higher expression of mono- and diubiquitin than the other samples.

The Coomassie blue staining suggests that the amount of protein loaded for each sample is equal.

We concluded that eukaryotic cells express at least two DUBs specifically in the nucleus. Furthermore, our results suggest strongly that the protein levels or activity of some DUBs may alter during cell cycling.

3.3 Investigation of human gut tissues

3.3.1 Background

We obtained snap frozen biopsies from inflammatory bowel disease (IBD) patients and controls from the Institute of Cell and Molecular Science, London School of Medicine and Dentistry, to analyze for differences in ubiquitin levels and DUBs. The disease samples included tissues from ulcerative colitis (UC) patients and patients with intermediate colitis (IC). There have been some speculations about the involvement of the ubiquitin system in IBD. In particular, levels of SMAD7 protein (a regulator of inflammatory responses) are known to be elevated in IBD despite normal levels of mRNA (47). This may reflect an alteration in its turnover by the ubiquitin-proteasome system. We wanted to find out if the levels of ubiquitinated proteins and/or DUBs were altered in the disease samples as compared to healthy controls.

3.3.2 Levels of active DUBs

Probing lysates using HAUbVME

We made lysates of the tissues before we could us them in our experiments. To do this, we used the Sample Grinding kit from Amersham Biosciences, which is designed for the processing of small tissue samples. After the homogenization, we quantified the protein levels by using the *DC* Protein Assay kit from Bio-Rad. We calculated the amount of lysate necessary to get 10 µg of proteins and

did the probe-reaction followed by SDS-Page and western blot for HA to detect probe-DUB conjugates. We ran two sets of samples of which one received the HAUbVME probe. Testing was performed blind to the clinical data (Figure 3.3.1).

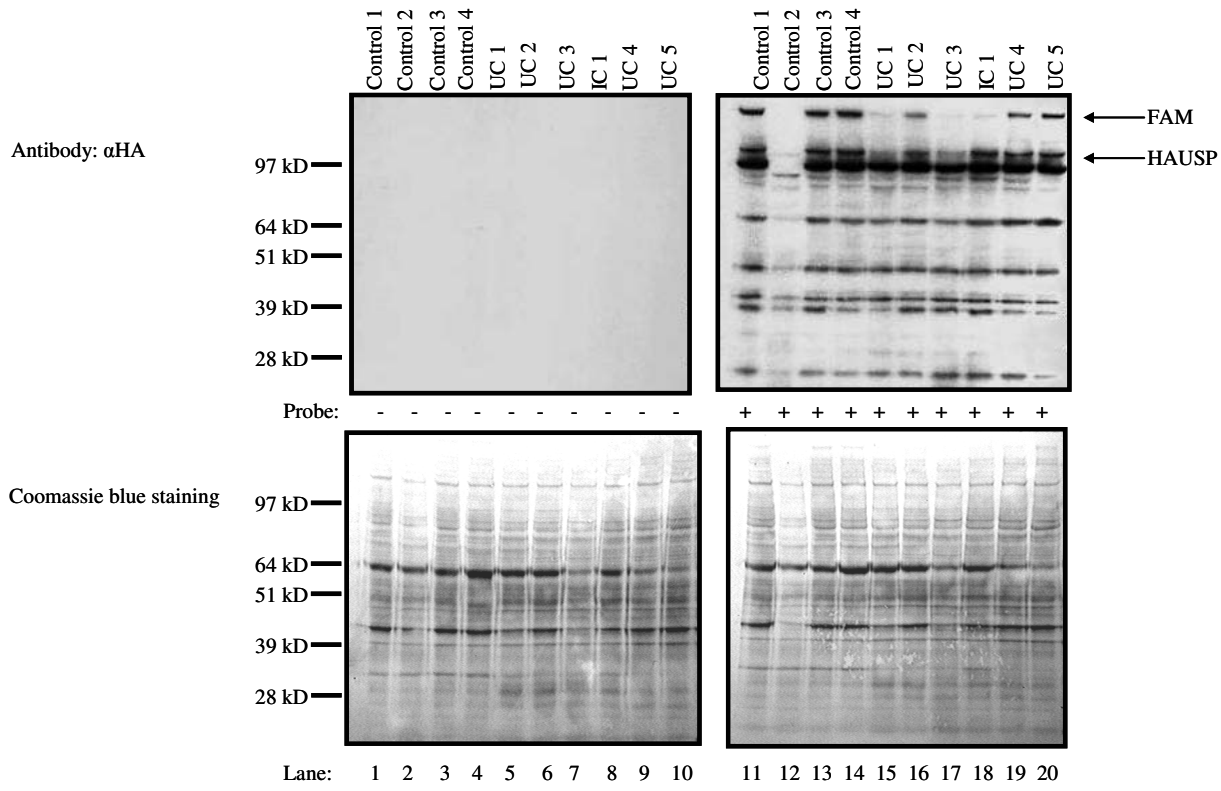

Figure 3.3.1: Western blot for HA. Left: Negative control for the probe-binding reaction. Right: Alteration of the expression of DUBs in IBD samples.

At first glance it seems like there are some DUBs missing in the 'Control 2' sample, but when we look at the Coomassie blue staining it is clear that the amount of protein loaded for this sample is less then for the others. The loading of the other samples looks fairly equal.

Interestingly, a band at 170 kD corresponding to FAM is absent in IBD samples (lanes 15, 17, 18) or is expressed at reduced levels (lanes 16, 19, 20) compared to controls. The levels of HAUSP are also reduced in two of the six IBD samples (lanes 15, 17). The set of samples that did not receive the HAUbVME probe was run as a negative control for the probe-binding reaction (lanes 1-

10). Since there are no visible bands this means that there were no unspecific binding of the antibodies.

Levels of Cytokeratin 19

We wanted to find out whether the relative lack of FAM and HAUSP in the IBD samples reflected loss of epithelium due to inflammation. To do this we ran a western blot for Cytokeratin 19. Cytokeratin 19 is a specific cytoskeletal structure of simple epithelia (65). The level of Cytokeratin 19 would therefore reflect the thickness of the epithelium in each biopsy (Figure 3.3.2).

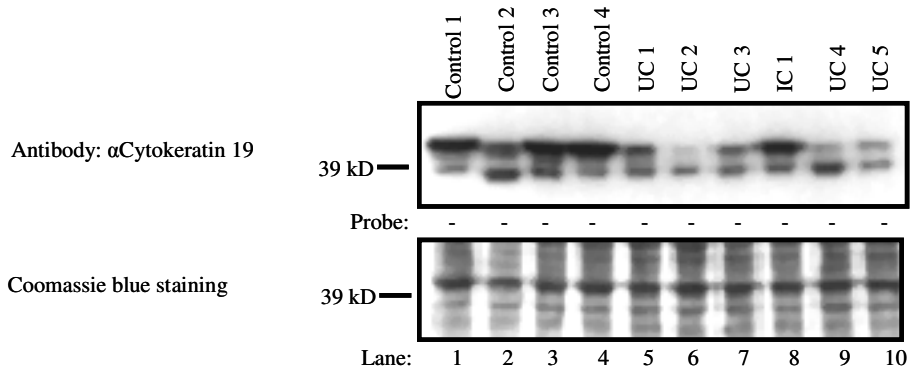

Figure 3.3.2: Western blot for Cytokeratin 19: Control for the amount of epithelial cells in each biopsy.

The IC sample (lane 8) shows the same intensity in Cytokeratin 19 levels as the control samples. The other IBD samples, however, show a decreased level of Cytokeratin 19 compared to the controls.

The gel implies that loss of epithelium due to inflammation is a contributing factor to the decreased levels of DUBs seen in the IBD samples. However, the fact that the IC sample (lane 8) shows a normal level of Cytokeratin 19 even though the levels of FAM was decreased for this sample in the HA blot, suggests that the molecular changes in IBD is more complex than just being a result of different amounts of epithelial cells in the samples.

3.3.3 Levels of ubiquitinated proteins

We also wanted to analyze the tissues for differences in levels of ubiquitinated proteins, so we did a second SDS-Page and ran a western blot for Ub. We used 20 µg of each sample (figure 3.3.3).

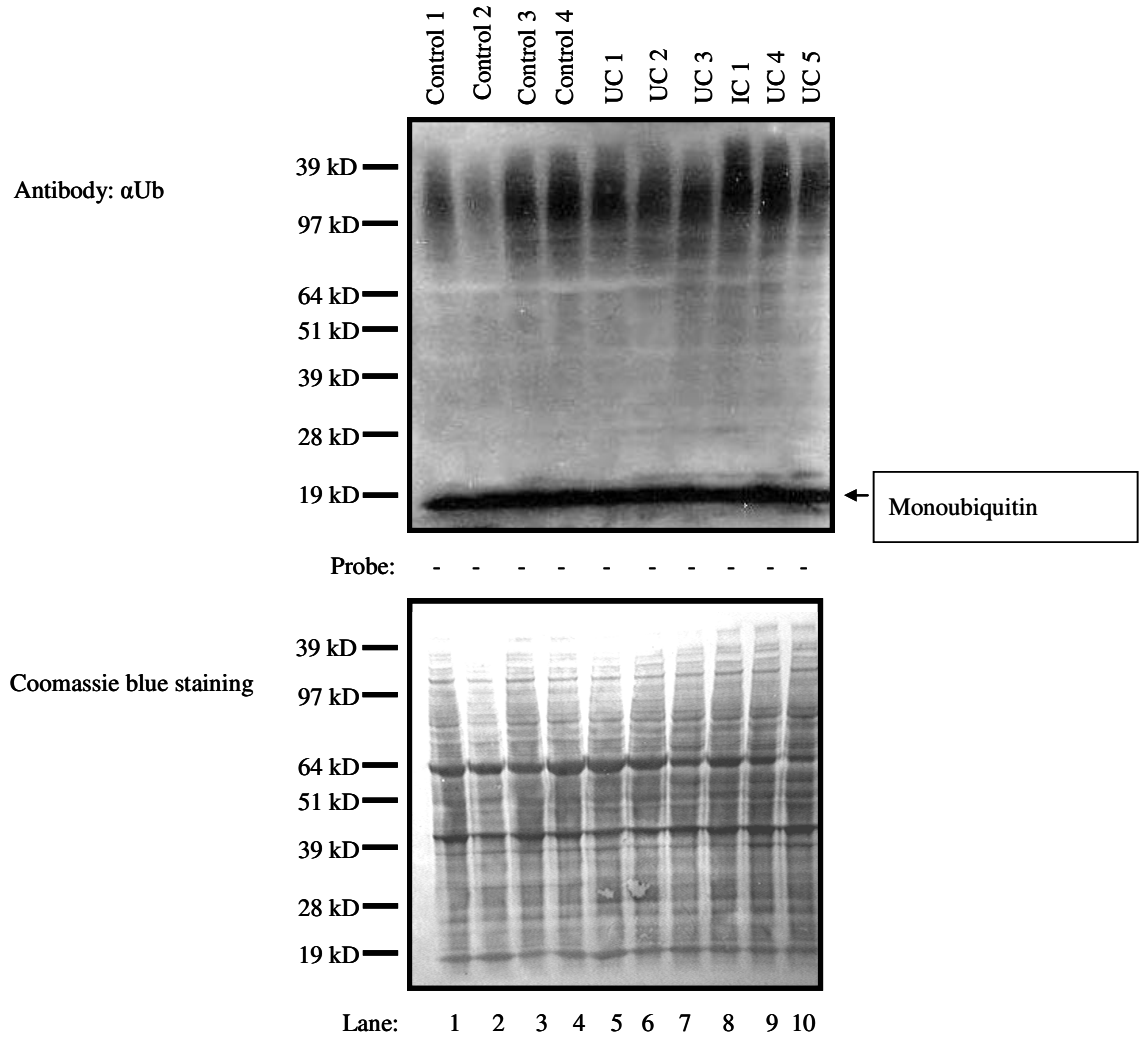

Figure 3.3.3: Western blot for Ub: Levels of ubiquitinated proteins and monoubiquitin in IBD samples.

The protein loading of the 'Control 2' sample (lane 2) is lower then for the rest of the samples in this experiment as well. It also seems like the protein load is a bit higher for the 'IC 1', 'UC 4' and 'UC 5' samples (lanes 8, 9, 10) compared to the rest of the samples. On this basis there seems to be no obvious differences between normal gut and IBD, neither in levels of monoubiquitin nor in levels of ubiquitinated proteins.

We concluded that the level of FAM is reduced in IC, otherwise the ubiquitin-proteasome system appears to be intact in IBD.

3.4 Investigation of human heart tissues

3.4.1 Background

Previous studies had suggested that heart failure is associated with perturbations in the ubiquitin proteasome system (58). In collaboration with scientists at Harefield Heart Science Center we examined whether alterations in the ubiquitin proteasome system were associated with changes in deubiquitinating activity.

We received snap frozen samples of human heart failure tissue and 'normal' transplant donor heart tissue from Harefield. The samples were divided into 3 groups as controls, ischemic heart disease (IHD) and dilated cardiomyophaty (DCM). The codes were as follows:

- Controls: U, A, F, I, Q, M
- IHD: B, C, H, N
- DCM: D, E, G, J, K, L, O, P, R, S, T, V

The heart failure group consisted of patients undergoing heart transplantation for advanced heart failure. All patients had undergone a prior assessment that included a medical history, clinical examination, two-dimensional echocardiography, cardiac catheterization, evaluation of haemodynamic function and coronary arteriography (Table 3.4.1).

Table 3.4.1: Demographics of patients used in this study.

	DCM (n=12)	IHD (n=8)*
Male:Female	18:2	7:1
Mean age (range)	42.4 (22-64)	53 (46-62)
Mean LVEDD (mm)	70.9 (59-90.9)	67.9 (58.5-84.2)
Mean LVESD (mm)	62.5 (50-81.8)	58.3 (50-74.2)
Mean Fractional Shortening	13% (6-20%)	13.4% (7-19%)
Mean Ejection Fraction	21% (11-38%)	24% (12-39%)
NYHA Class 3:4	10:2	7:1
Diuretics	16	6
ACE Inhibitors	9	8
Inotropes	6	2
Digoxin	4	6
Nitrates	2	6
ß-Blockers	2	0

*We received only 4 of these 8 samples.

The control group consisted of donors used for transplantation (Mean age 33.5, age range 2-51 years). Donor hearts were assessed by transoesophageal echocardiography prior to retrieval and all were judged to have an ejection fraction greater than 55%. Causes of death were subarachoid hemorrhage, road traffic accident, intracranial bleed, asthma and one sample was obtained from heart-lung transplantation as a result of cystic fibrosis. Hearts were reassessed at one week after transplantation and all had good ventricular function.

3.4.2 Levels of active DUBs

Probing lysates using HAUbVME

We first had to use a homogenizer to make cytosolic lysates of the tissue samples. We then quantified the amounts of proteins in the samples and calculated the amount of lysates necessary to get 10 µg of each sample. We ran the probe-binding reaction on two sets of samples of which one set received the HAUbVME probe while the other did not. The probe-binding reaction was followed by SDS-Page electrophoresis and western blots for HA. Since there were as many as 22 different samples we had to split the samples in two. We mixed the order of the samples so that all the different gels contained samples from all three groups. Testing was performed blind to the clinical data (Figure 3.4.1).

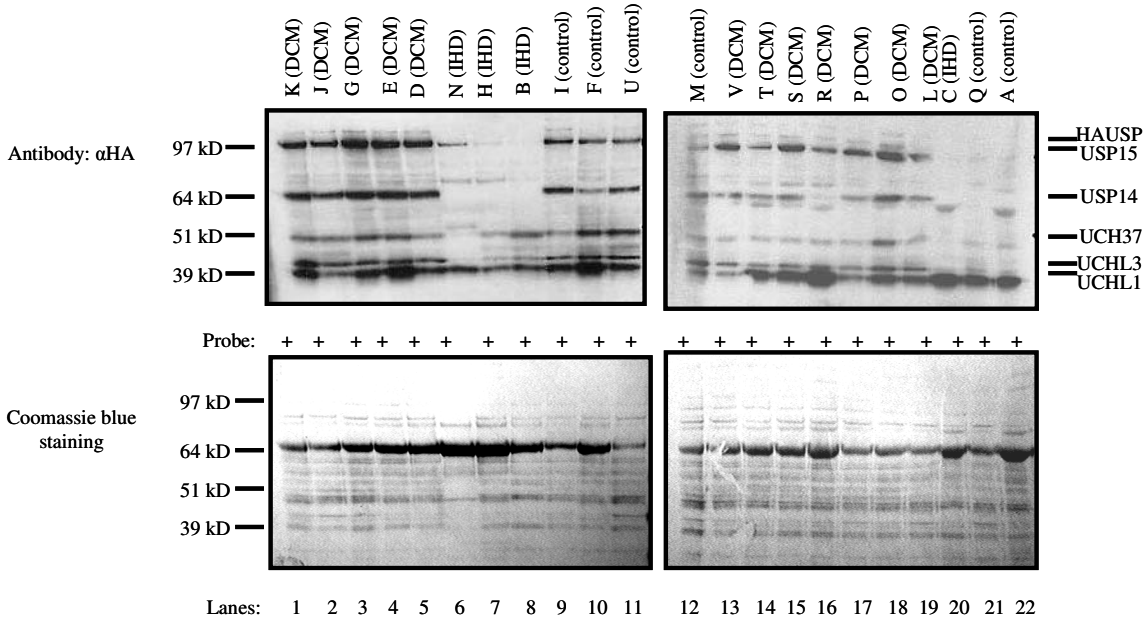

Figure 3.4.1: Western blot for HA: Alteration of the expression of DUBs in IHD samples. Left: First half of the heart tissue samples. Right: Second half of the samples.

It is apparent that the amounts of active DUBs are decreased in the IHD samples compared to the DCM samples and the controls. HAUSP and USP14 are entirely absent in the IHD samples (lanes 6, 7, 8, 20). In addition USP15, UCH37 and UCH-L3 are greatly diminished. The level of UCH-L1 is unchanged in the IHD samples compared to the DCM and control. The DCM samples look fairly similar to most of the healthy controls (lanes 9-12).

Two of the control samples, however, show the same decrease in levels of active DUBs as the IHD samples (lanes 21, 22).

Coomassie blue staining reviled that protein loading was equivalent in each well. However, sample N (lane 6) showed an unusual profile with staining concentrated at 64 kD. When we quantified the total amount of protein for each sample, the protein or, more likely, proteins behind this 64 kD band would have constituted the lion's share of the quantity measured. As a result of this the amount of other proteins would be less compared to the other samples when we loaded 10 µg of proteins. It might therefore be that the amount of proteins loaded for sample 'N' in fact was a bit less then for the rest of the samples. We chose, therefore, not to include sample 'N' for our analysis.

We repeated this experiment for both half's of the samples and the results were reproducible (data not shown).

As a control for the probe-reaction we ran a second set of samples without including the HAUbVME probe in the reaction mixture. Any binding of the antibodies to these samples would be unspecific (Figure 3.4.2).

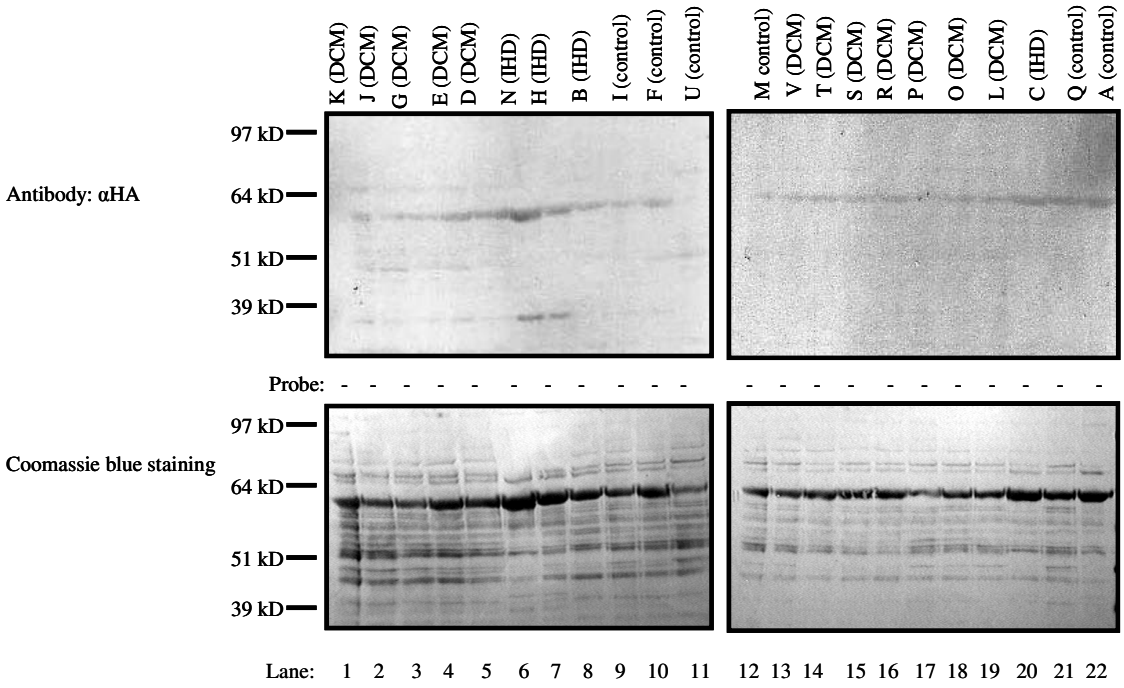

Figure 3.4.2: Western blot for HA: Negative control for the probe-binding reaction. Left: First half of the heart tissue samples. Right: Second half of the samples.

There is some unspecific binding of proteins, but this does not interfere with our findings for the IHD samples. It seems like the proteins, or some of the proteins, in the dense band below the 64 kD mark in the Coomassie blue staining bind the antibodies unspecific.

Analysis of three housekeeping proteins

We wanted to confirm that the differences in DUB levels found between IHD, DCM and healthy tissues were not due to differences in loading. Our Coomassie blue stainings strongly suggest that protein levels were identical between wells (perhaps with the exception of sample 'N'). To validate this further we wanted to use western blotting to detect a protein that is expressed equally in IHD,

DCM and controls. Such proteins are called housekeeping proteins, and they are products of genes that are constitutively and stably expressed at high levels in almost all tissues and cells. We decided to run western blots for α-tubulin, which is a well-known housekeeping protein. Tubulin is the protein that makes up microtubules. Microtubules are assembled from dimers of α- and ß-tubulin. Microtubules are part of the cytoskeleton within a cells cytoplasm and contribute to the cells structural support. Microtubules also have other functions, such as being part of the mitotic spindle used by eukaryotic cells to segregate their chromosomes correctly during cell division (66). We used 10 µg of each sample (Figure 3.4.3).

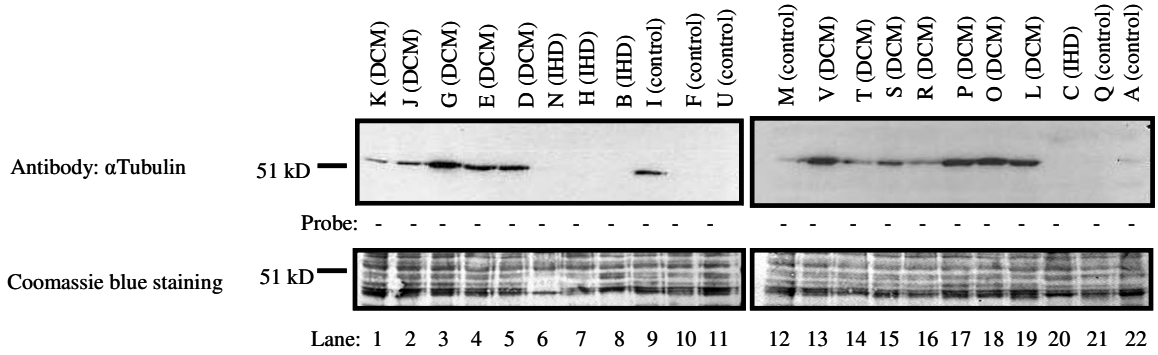

Figure 3.4.3: Western blot for Tubulin: Loading control. Left: First half of the heart tissue samples. Right: Second half of the heart tissue samples

It turned out that tubulin is not a good housekeeping protein for these samples as all the IHD and five of six control biopsies do not express tubulin, at least not in detectable amounts. Some of the DCM samples also show a low expression of tubulin (lanes 1, 2, 14, 15, 16).

Because of these findings we decided to run a western blot for actin, which is another well-known housekeeping protein. Actin is a small protein within cells that assembles into long filaments. A tangle of cross-linked actin filaments fills the cytoplasm of all cells forming the cytoskeleton that gives the cell shape and form and provides a scaffold for organization. Actin also forms the ladder on which myosin climbs, providing the infrastructure for muscle contraction (67). We ran 10 µg of each sample (Figure 3.4.4).

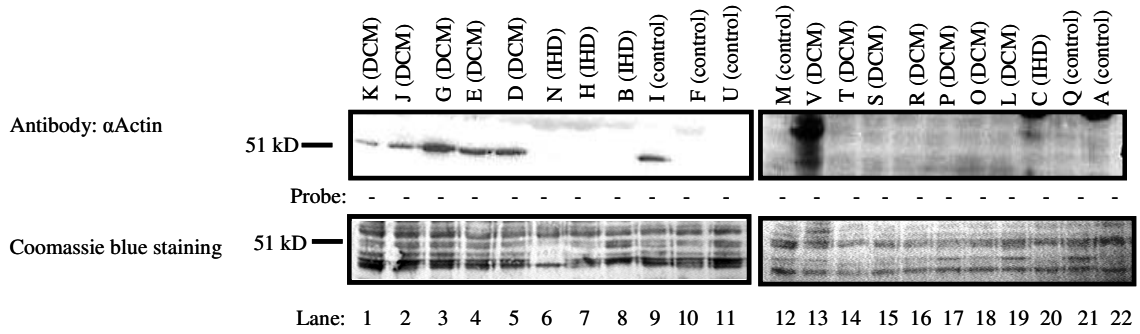

Figure 3.4.4: Western blot for Actin: Loading control. Left: First half of the heart tissue samples. Right: Second half of the heart tissue samples.

The actin antibody did not give staining either. For the first half of the samples it is just the IHD samples and the controls that do not show expression of actin. When we ran the second half of the samples however, all samples except 'V', which was a DCM sample, showed lack of actin. There were some bands in addition to the actin band in these experiments, so there was some unspecific binding of one or both of the antibodies.

We were determined to find a good housekeeping protein to confirm equal loading of the samples. We therefore decided to try a third housekeeping protein and this time we chose glyceraldehyde-3-phosphate dehydrogenase (GAPDH). GAPDH is a catalytic enzyme involved in glycolysis. Besides its function as a catalytic enzyme within the cytoplasm, GAPDH is also involved in the early steps of apoptosis. Recent evidence suggests that mammalian GAPDH is also involved in some other processes such as membrane fusion, microtubule bundling, phosphotransferase activity, nuclear RNA transport and DNA replication (68). We used 10 µg of the samples (Figure 3.4.5).

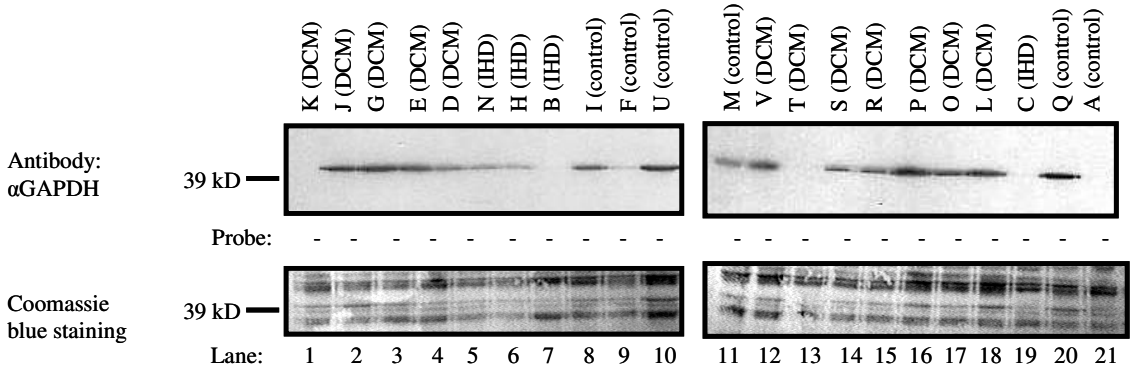

Figure 3.4.5: Western blot for GAPDH: Loading control. Left: First half of the heart tissue samples. Right: Second half of the heart tissue samples.

It turned out that not even GAPDH is expressed in all the tissue samples. It seems that the lack of GAPDH is quite random among these samples; in each group some samples express the protein while others do not.

We concluded from this data and from the Coomassie-staining that the changes in DUB activity observed in IHD are not simply due to variations in sample loading. However, IHD and DCM were characterized by multiple molecular changes, which might reflect their complexity.

3.4.3 Levels of ubiquitinated proteins

To investigate whether or not the levels of ubiquitinated proteins are altered in heart failure compared to control tissues we ran a western blot for Ub. We used 15 µg of each sample (Figure 3.4.6).

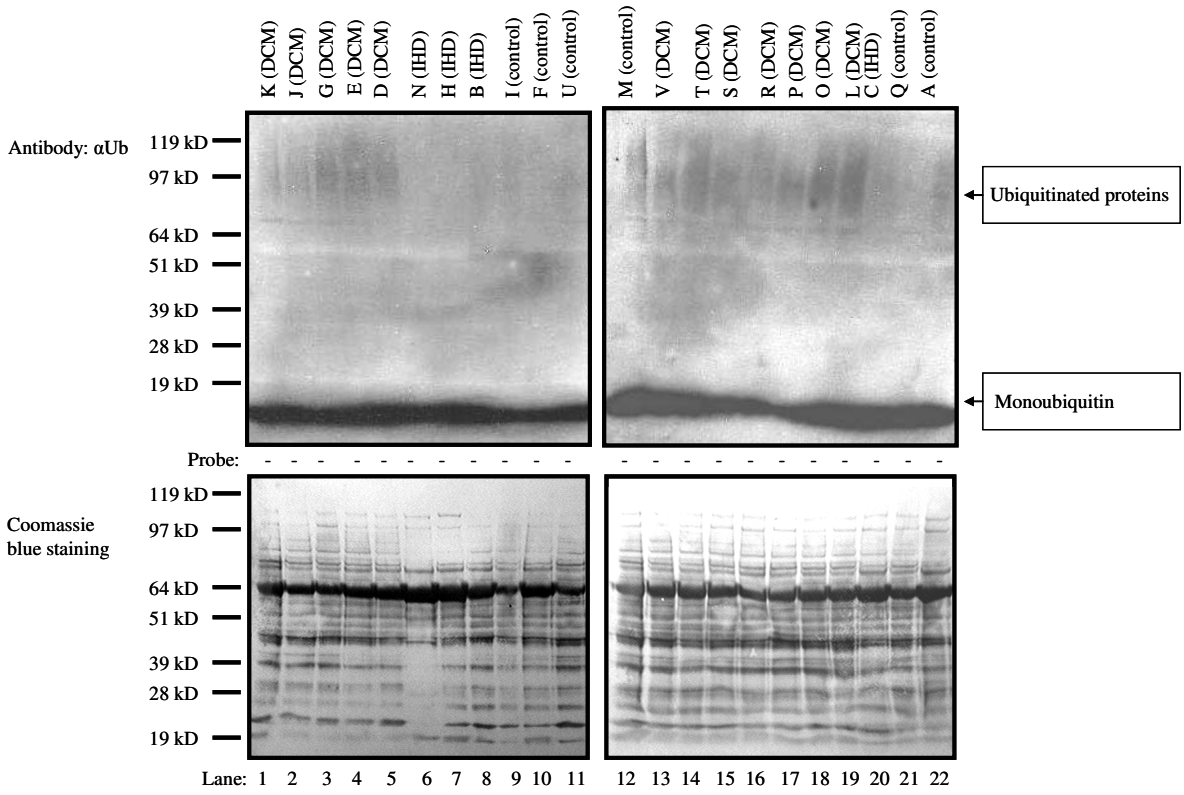

Figure 3.4.6: Western blot for Ub: Increased amounts of ubiquitinated proteins in DCM samples compared to IHD samples and controls. Left: Second half of the heart tissue samples. Right: First half of the heart tissue samples.

The levels of ubiquitinated proteins are clearly higher for the DCM samples compared to IHD and control tissues. An exception may be sample 'M' which is a control sample (lane 12). The levels of ubiquitinated proteins in this sample look more similar to the DCM samples then to the other controls. The levels of monoubiquitin seem to be fairly equal for all the samples.

The Coomassie blue staining may suggest that the loading of sample 'N' again is a bit lower then for the other samples. Otherwise the protein levels seem to be relatively equal.

3.4.4 Are HAUSP protein levels reduced in IHD?

An interesting question was whether the absence of probe-binding in IHD was due to inactivation of DUBs or reduction in expression levels.

We hypothesized that the absence of a probe binding by HAUSP in the IHD samples reflected its reduced expression. To address this idea we ran a western blot for HAUSP using 10 µg of each

sample. We also wanted to confirm that the 100 kD band in fact corresponded to HAUSP (Figure 3.4.7).

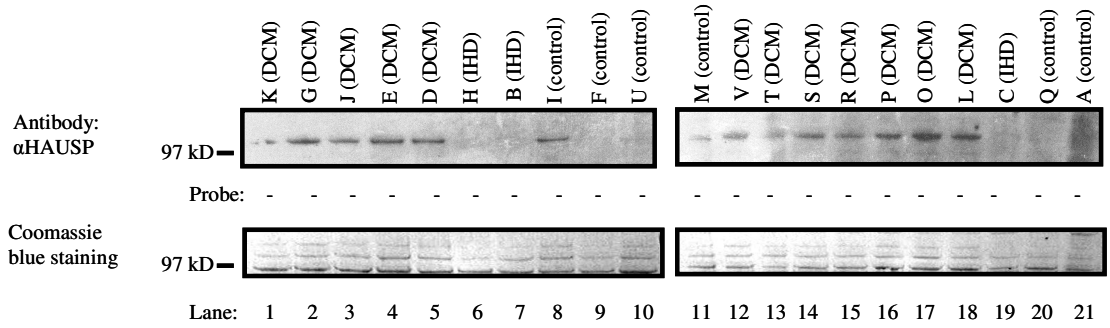

Figure 3.4.7: Western blot for HAUSP: The blot confirms that the absence of HAUSP in the probe-binding analysis was due to reduced expression of this DUB. The blot also confirms of that one of the missing DUBs in the IHD samples is HAUSP. Left: First half of the heart tissue samples (at this point we had run out of lysate for sample 'N', so this sample was not included in this Western blot). Right: Second half of the heart tissue samples.

None of the IHD samples seem to contain active HAUSP. This is also the case for four of the six control samples (lanes 9, 10, 20, 21). All the DCM samples show quite equal levels of the DUB. The Coomassie blue staining shows that there were equivalent amounts of proteins in all the samples.

We concluded that IHD is associated with reduced expression of HAUSP compared to DCM.

3.4.5 Induction of hypoxic effects in HUVEC

Probing lysates using HAUbVME

We wanted to explore the mechanism that caused decreased levels of active DUBs in IHD. Since IHD is characterized by reduced supply of oxygen and nutrients to the heart muscle we wanted to find out if a decreased supply of oxygen could cause the effects we had seen. We decided to use HUVEC in this experiment, since endothelial cells are important participants in heart function and hence heart dysfunction. We seeded HUVEC in small dishes and when they were about 90% confluent we exposed them to an environment with only 1% oxygen for 8, 16, 24 and 96 hours respectively. Duplicate dishes were kept at normal oxygen levels as controls.

After lysing the cells and quantifying the amount of proteins in each sample we ran the probe-binding reaction and western blot for HA to detect probe-DUB conjugates. We used 10 µg of each sample (Figure 3.4.8).

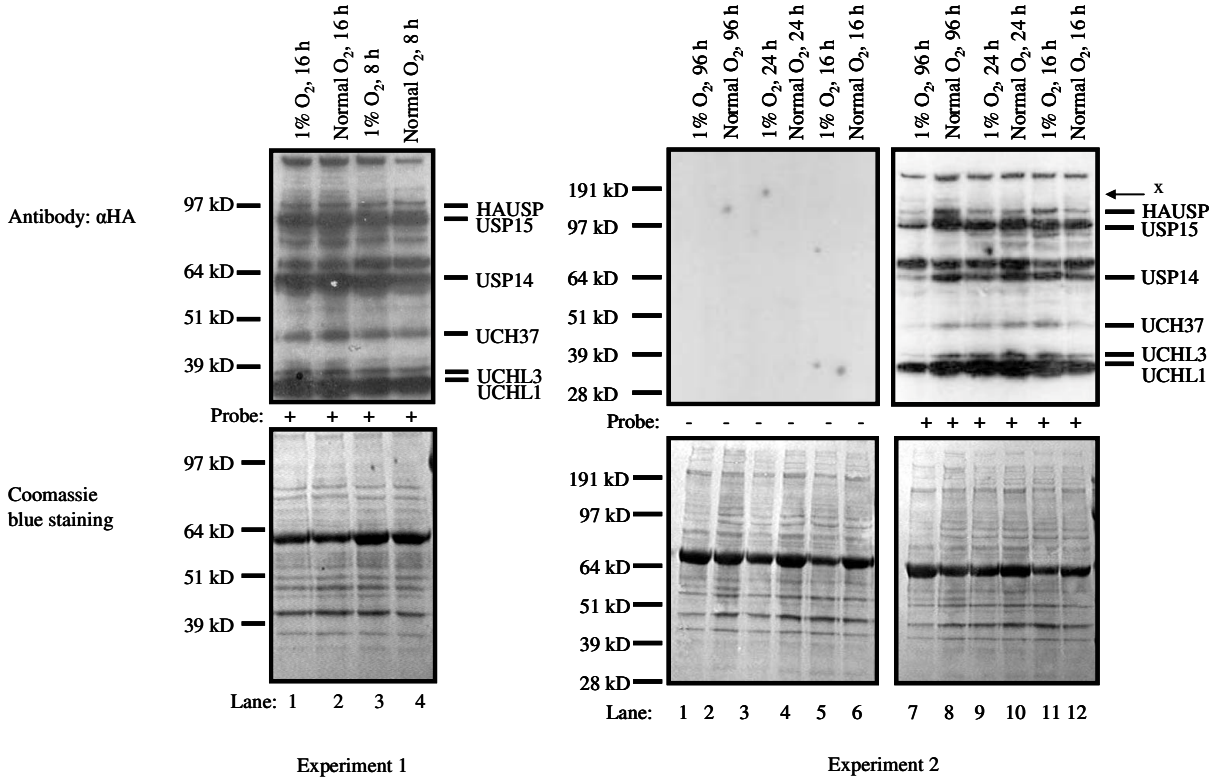

Figure 3.4.8: Western blot for HA: The effect of hypoxia on the level of active DUBs in endothelial cells.

There are no obvious differences in DUB activity between the samples grown under normal oxygen conditions and those grown under hypoxic conditions for 8, 16 and 24 hours. However after 96 hours in an environment with only 1% oxygen the activity or expression of some of the DUBs is decreased compared to cells grown under normoxic conditions (experiment 2, lane 7). The level of HAUSP is clearly lower in the hypoxic sample as compared to the normoxic sample. The DUBs corresponding to USP15, USP14, UCH37 and UCH-L3 are also weak compared to the controls.

It also seems like the level of UCH-L1 is somewhat lower for the '1% O_2, 96 h' sample then for the other. Another interesting feature with the two 96 hour samples in the HA blot is the appearance of a new band labeled x (experiment 2, lanes 7, 8). There are no bands for the samples that did not receive the probe, which means that there is no unspecific binding of the antibodies. The

Coomassie blue staining suggests that the loading is equal for all the samples. We repeated this experiment and the results were reproducible (data not shown).

Loading control

To further control the loading we decided to run a western blot for GAPDH using 20 µg of each sample (Figure 3.4.9).

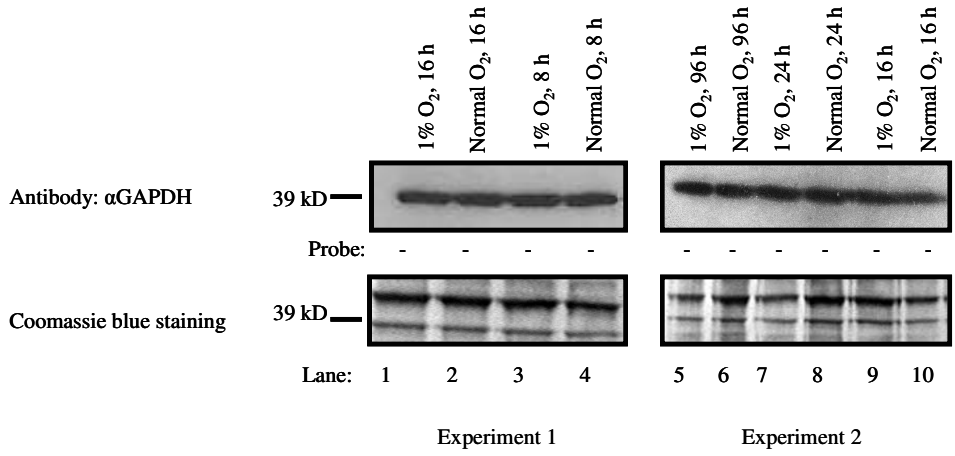

Figure 3.4.9: Western blot for GAPDH: Loading control.

GAPDH levels were equivalent for each of the samples.

The Coomassie blue staining also suggests that the loading was equal for all the samples.

Densitometry analysis

To validate our findings further we did a densitometry analysis on some of the bands (Figure 3.4.10) and standardized them against GAPDH (Figure 3.4.9). This confirmed that prolonged hypoxia reduces levels of HAUSP, USP14, UCH37 and UCH-L3 in HUVEC.

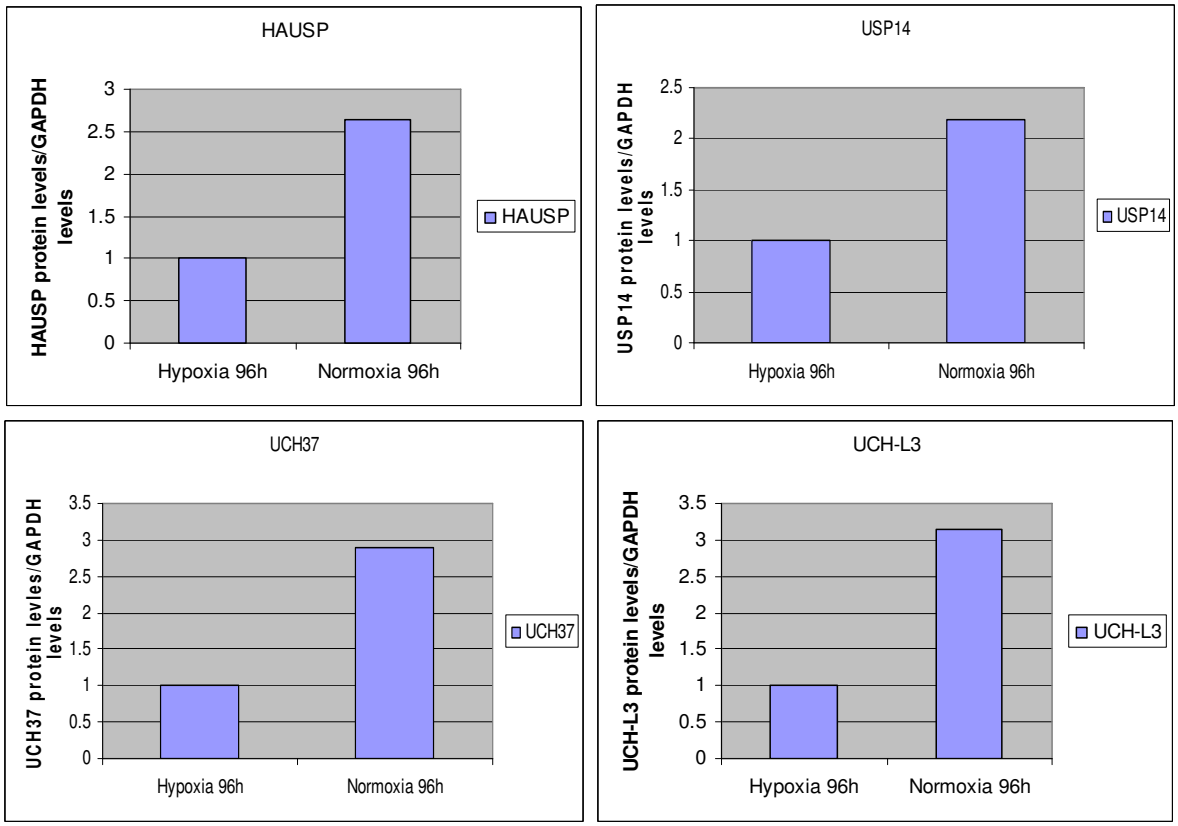

Figure 3.4.10: Densitometry data showing the decrease in levels of DUBs in HUVEC exposed to a hypoxic environment for 96 hours compared to control samples grown in normoxia.

Level of ubiquitinated proteins

We also wanted to see if the exposure of HUVEC to hypoxia would have an effect on the amount of ubiquitinated proteins. We therefore ran a second western blot, this time for Ub. We used 20 µg of the samples (Figure 3.4.11).

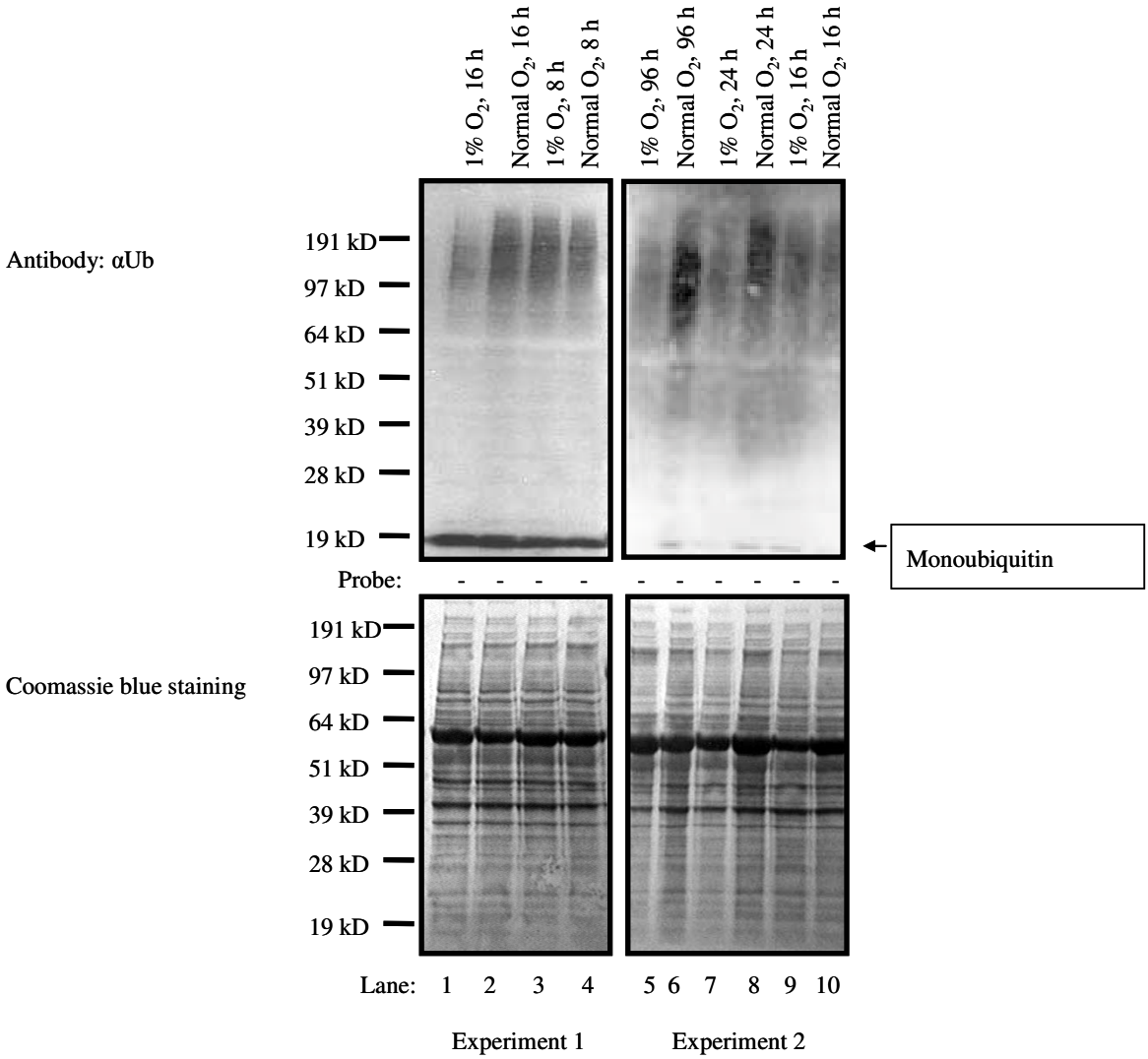

Figure 3.4.11: Western blot for Ub: The effect of hypoxia on the levels of ubiquitinated proteins.

There are no obvious differences between the normoxic samples and the sample grown under hypoxic conditions for 8 and 16 hours (experiment 2) (lanes 3, 4, 9, 10). However, the amount of ubiquitinated proteins is clearly lower for the samples grown under conditions of just 1% oxygen for 16 hours (experiment 1), 24 and 96 hours compared to the controls (lanes 1, 2, 7, 8, 5, 6).

The levels of monoubiquitin vary between the samples in experiment 2 but there is not an obvious pattern. Some of the ischemic samples and some of the controls seem to have lower levels of monoubiquitin.

The Coomassie blue staining shows that the quantity of protein in the 24 hours hypoxic sample (lane 7) might be a bit less then for the other samples. However, the amount of protein in the '1% O_2, 96 h' sample (lane 5) and the '1% O_2, 16 h' (experiment 1, lane 1) is similar to the control samples and it is obvious that the amount of ubiquitinated proteins is decreased in these samples. This means that the reduction in the amount of ubiquitinated proteins seen for the three hypoxic samples is due to the environment in which these cells where grown and not the amount of protein loaded.

We conclude that prolonged hypoxia causes reduced activity of several DUBs and also influences total levels of polyubiquitinated proteins in HUVEC.

3.5 The effect of hypoxia and IL-1 on the translocation of NF-κB

3.5.1 Background

The transcription factor NF-κB is tightly regulated by the ubiquitin proteasome system (see introduction). We wanted to answer the question whether changes in the ubiquitin-proteasome system seen in hypoxia correlate with perturbations in NF-κB translocation. Specifically, we hypothesized that reduction in ubiquitin-proteasome activity under hypoxic conditions may inhibit NF-κB function. p65 is one of the proteins in the NF-κB family (60). We used an antibody against p65 in these experiments. It is well-known that IL-1 causes translocation of p65 into the nucleus (4). We tested the effects of hypoxia on the translocation of p65 into the nucleus in response to IL-1.

3.5.2 The effect of hypoxia and IL-1 on the translocation of p65

We plated out HUVEC on glass coverslips in eight small dishes (35 mm diameter). Two of these dishes were kept in normoxic conditions as control samples. The other dishes were kept in duplicates for different amounts of time at hypoxic conditions (1% O_2). Each duplicate dish received 20 ng/ml IL-1 (Human IL-1 (Peprotech)) for the final 30 minutes of the incubation (Table 3.5.1).

Table 3.5.1: Experimental set up to test NF-κB activity in hypoxia.

Dish number	Preconditioning	Incubation time (hours)	Stimulation (20 ng/ml IL-1)
1	Hypoxia	2	-
2	Hypoxia	2	30 minutes
3	Hypoxia	4	-
4	Hypoxia	4	30 minutes
5	Hypoxia	6	-
6	Hypoxia	6	30 minutes
7	Normoxia	N/A	-
8	Normoxia	N/A	30 minutes

After the incubation the cells were permeabilized, stained with primary (rabbit αp65) and secondary (goat αrabbit- alexafluor 568) antibodies and mounted onto glass slides. Pictures were taken using confocal microscopy (Figure 3.5.1).

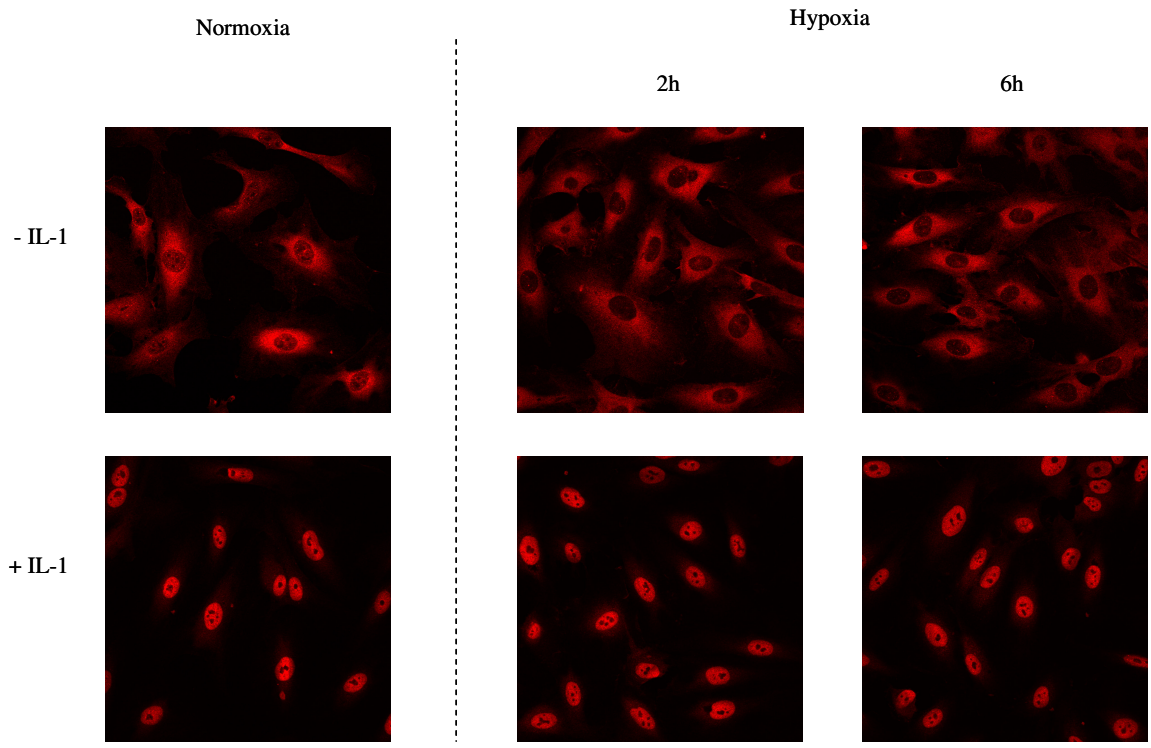

Figure 3.5.1: The effect of IL-1 and hypoxia on the location of p65 within endothelial cells. Red color indicates p65.

The normoxic sample without IL-1 shows that p65 is uniformly distributed in the cytoplasm. In the other control sample however, where IL-1 has been added, p65 has clearly been translocated to

the nucleus. After 2 and 4 hours in the hypoxic environment without IL-1 stimulation, p65 is still located in the cytoplasm. Addition of IL-1 to these samples gives translocation of p65 into the nucleus to the same extent as for the control sample.

Before doing this experiment we did a preliminary experiment with longer time points (19 hours and 24 hours). This experiment suggests that prolonged hypoxia does not inhibit the translocation of p65 into the nucleus in response to IL-1 (data not shown).

Overall my experiments indicated that hypoxia does not inhibit the translocation of p65 to the nucleus in response to IL-1. However, the last experiment revealed something interesting. Some NF-κB is always transported constitutively into the nucleus even in the absence of an activating signal. It appears that hypoxia inhibits this constitutive translocation (Figure 3.5.2).

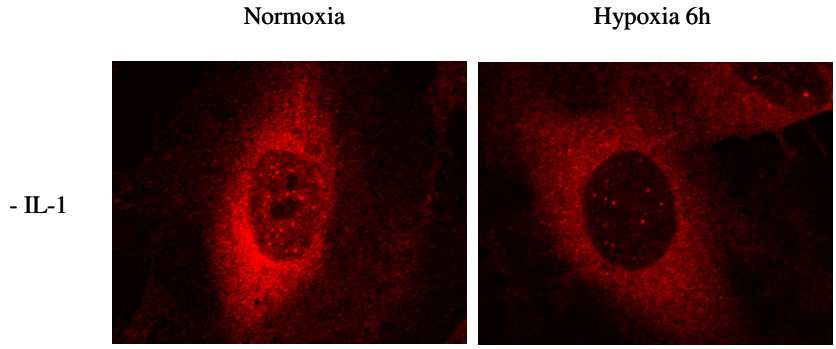

Figure 3.5.2: Confocal images showing the inhibition of the constitutive translocation of p65 in response to hypoxia.

It is quite clear that the level of p65 in the nucleus is lower in the cell grown in a hypoxic environment than in the cell grown under normoxic conditions. However, to confirm that this observation applied to a representative selection of cells, we quantified the level of p65 in the nucleus and in the cytoplasm for a collection of both normoxic and hypoxic cells. By using the GraphPad Prism Version 4.00 software, we made a graph to visualize the level of p65 in the nucleus for the different samples (Figure 3.5.3).

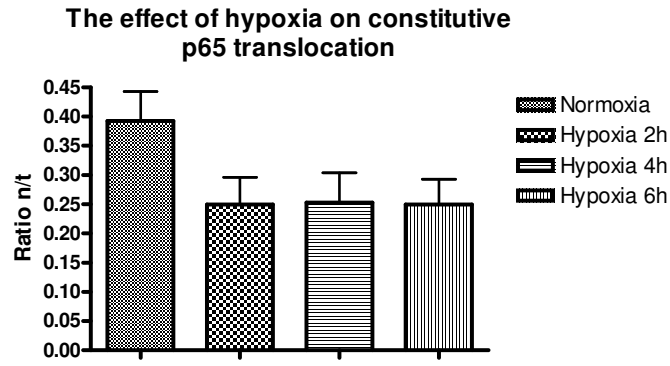

Figure 3.5.3: The effect of hypoxia on constitutive p65 translocation. n = level of p65 in the nucleus, t = total (level of p65 in the nucleus + level of p65 in the cytoplasm).

The graph confirms that the constitutive translocation of p65 into the nucleus is inhibited when HUVEC are cultured in an environment with only 1% oxygen.

4. DISCUSSION

4.1 Expression patterns of novel 50 kD and 60 kD DUBs identified in T-cells

4.1.1 Experiments to reveal the identity of DUBs found in proliferating CD4+ T-cells

The western blot for HA showed that neither the band of interest at 70 kD (60 kD without probe) nor the one at 60 kD (50 kD without probe) in the lysate from the CD4+ T-cells was present in the lysates from the HEK 293T cells transfected with DUB-2 DNA. Furthermore, neither of these DUBs reacted with anti-DUB-2 antibodies. This strongly suggests that neither of these proteins corresponds to DUB-2.

The HA antibody was not able to pick up the signals from DUB-2 in the transfected cells very well. The DUB-2 antibody however, gave dense bands for both DUB-2 and DUB-2 cs. This indicates that the DUB-2 antibody was more sensitive than the HA antibody. The HA antibody was sensitive enough to pick up the signals from the two DUBs of interest in the T-cell lysate, but the more sensitive DUB-2 antibody did not give any signals at all in the CD4+ lysate. Overall my experiments revealed that neither of the two proteins found at 60 kD and 70 kD respectively in the lysate from the proliferating CD4+ T-cells is DUB-2.

An inactive form of DUB-2 mutated at the catalytic site (DUB-2 cs) did not bind the probe. For active DUB-2 however, some of the forms were shifted to a higher level when combined to the probe, while other forms seemed to be unable to bind the probe. It was particularly the low molecular weight forms of DUB-2 that seemed unable to bind the probe. An explanation may be that these were degradation products of DUB-2 where the catalytic site was damaged or lost.

4.1.2 Are the DUBs found in proliferating CD4+ T-cells also found in HUVEC?

When we ran the western blot for HA we could clearly see the band at about 70 kD in the sample from the proliferating CD4+ T-cells, but the band at 60 kD was faint. The T-cell lysate had been stored for quite some time, so a possible explanation for the low intensity of the 60 kD band might

be that the proteins in this sample had started to be broken down. Since neither of the bands appeared in the HUVEC samples, neither in the lysate from the proliferating nor the non-proliferating cells, it is reasonable to assume that the two proteins of interest are not present in HUVEC.

There was a band at approximately 62 kD in a sample of proliferating HUVEC, which was not present in the other HUVEC samples. The Coomassie blue staining showed a huge amount of protein below this band. One of my colleagues previously found that serum proteins apparently 'react' with the HAUbVME probe and yield a band at 62 kD, i.e. the 62 kD band in HUVEC was at the same place as one of the bands you get when you run samples containing serum alone (Figure 4.1.1).

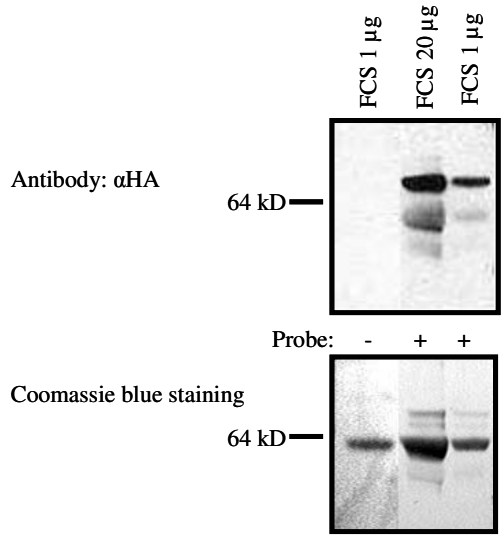

Figure 4.1.1: Western blot for HA: Artifacts created by proteins in serum. Data obtained from Karine Enesa.

Because of these earlier findings we decided not do go any further with this band and just assume that it was caused by non-specific binding of the probe to proteins in serum.

4.2 Identification of DUBs in nuclear lysates in different stages of the cell cycle

4.2.1 Levels of active DUBs

DUBs with specific nuclear and cytoplasmic localization

We identified a DUB of approximately 85 kD (95 kD combined with probe; x) that was only present in nuclear lysates. Another DUB of approximately 90 kD (100 kD with probe; w) was also clearly denser for the nuclear samples. Nobody has ever looked for nuclear specific DUBs before, so the presence of these bands is very interesting. Conversely, FAM was only visible in the cytosolic lysate, which means that this is a cytosol specific DUB. This might suggest that deubiquitination of its target ß-catenin occurs in the cytoplasm but not in the nucleus, which may have implications for Wnt signalling. Overall, we conclude that many DUBs are likely to be regulated at least partly by their specific localizations in the cell.

DUBs in mitosis

A DUB of 40 kD (y) was present in two of the nuclear samples in addition to the cytosolic one. Since we do not know the identity of this DUB it is difficult to draw any conclusions from this finding. It is interesting though that this DUB was just present in the G1 and asyncronized cells and not in cells in M or S phase, which suggests that deubiquitination may play an important part in mitosis. Our data suggests that cell division is associated with alterations in the activity of a 40 kD DUB.

Our results also suggested that a DUB (z) was missing from ‘S, IR’, ‘S-Low-IR’ and ‘S-phase’ samples, however we were not confident about this particular result because of technical considerations (see ‘Results’ section 3.2.2).

Overall, our findings are potentially important in the context of cancer, a disease that culminates from defective regulation in cell division. A number of reports have linked ubiquitination to cell cycle regulation. For example, our collaborator, Dr. Jo Morris, is working on an E3 ligase called

BRCA1 that regulates DNA repair and cell cycle checkpoint control. BRCA1 is localized to the nucleus, where it targets DNA repair proteins for ubiquitination. Loss of BRCA1 is thought to cause genomic instability, which predispose towards breast cancer. It is tempting to speculate that deubiquitination may also affect the stability of DNA repair proteins and other nuclear proteins and, therefore, control of genomic stability or cell cycle control.

4.2.2 Levels of ubiquitinated proteins

The levels of ubiquitinated proteins were clearly higher for the nuclear samples than for the cytosolic. The 'S-Low-IR' sample seemed to have the highest level of ubiquitinated proteins. This partly correlates with our colleagues' previous findings that S-phase cells, particularly S-phase cells treated with DNA damaging agents, produce ubiquitin conjugates at an increased rate. However, we did not see an increased amount of ubiquitinated proteins in the other S-phase samples compared to the G1-, M-phase- and asyncronized samples.

4.3 Investigation of human gut tissues

4.3.1 Levels of active DUBs

The IBD samples consisted of one intermediate colitis (IC) sample and five ulcerative colitis (UC) samples. There was an obvious decrease in the level of FAM in the IC sample and 2/5 of the UC samples. The other UC samples also seemed to have a small decrease in the intensity of this band. Overall, IBD samples showed decreased levels of FAM.

FAM stabilizes ß-catenin in one of two ways: It is possible that FAM regulates the degradation of ß-catenin by preventing its ubiquitination, the alternative is that FAM inhibits the binding of APC and/or Axin to ß-catenin and hence represses the function of this/these proteins (31). Loss of FAM could cause a reduction in ß-catenin levels. This is interesting because it has been shown that cadherin-catenin complexes are important for the maintenance of epithelial architecture (32). It is plausible, therefore, that alterations in FAM levels might contribute to the changes in epithelial architecture that are characteristic of IBD.

UC was also associated with decreased levels of HAUSP. This DUB is known to stabilize MDM2, which is the ubiquitin ligase for p53. Hence the effect of HAUSP is to destabilize p53 (22). Loss of HAUSP would be expected to cause elevation in p53 levels leading to apoptosis. This is consistent with the data in a recant paper suggesting that p53 levels are increased in UC (42). We hypothesize that the observed reduction of HAUSP in UC might contribute to epithelial apoptosis, which is an important marker of this disease.

A caveat to these conclusions is that Cytokeratin 19 was reduced in UC suggesting that these samples may have experienced loss of epithelial cells due to inflammation. We cannot therefore state with certainty that the decreased levels of FAM and HAUSP seen for these samples were caused by down-regulation or inactivation of these DUBs in UC. The IC sample however, showed normal levels of Cytokeratin 19 even though the levels of FAM was clearly decreased compared to the control samples in the HA blot. This suggests that FAM is indeed down regulated or inactivated in IC.

4.3.2 Levels of ubiquitinated proteins

Our results from analysis of ubiquitinated proteins suggest that the rate of ubiquitination and deubiquitination stays normal in IBD. The levels of monoubiquitin were unaffected in the IBD samples, and this correlates well with the findings of normal levels of ubiquitinated proteins.

4.4 Investigation of human heart tissues

4.4.1 Levels of active DUBs

Probe-binding assay

Ischemic heart disease (IHD) had an obvious association with the activity or the expression of certain DUBs. HAUSP and USP14 were entirely absent in all IHD samples. In addition, the intensity of the bands that corresponded to USP15, UCH37 and UCH-L3 were greatly diminished.

In addition 2/6 of the transplant donor controls showed the same decrease in levels of active DUBs as the IHD samples. One possible explanation may be that these control donor hearts

experienced ischemia during transplantation or transportation. Normal human myocardial tissue is extremely difficult to obtain and can only be obtained from donors, and they vary greatly in their ischemic times. Our collaborators at Harefield have done a study where they compared donors that were used for transplantation and those that were unused due to long ischemic times. They found pronounced apoptosis in the unused tissues, which suggests that the ischemic time influences the state of the tissue. We do not have the detailed history of the individual hearts so we cannot compare the ischemic time for these samples with the other control samples.

HAUSP interacts in a complex fashion with the tumor suppressor protein p53 (14,22). A decrease in the levels of HAUSP might lead to increased amounts of p53, a known inducer of apoptosis. This is very interesting as our collaborators have discovered increased level of apoptosis in heart tissue exposed to prolonged ischemia.

UCH37 is most likely an integral subunit of the 26S proteasome. It is thought to be responsible for the majority of the deubiquitination activity to the proteasome, but it is also assumed that other DUBs may take its place when the enzyme is missing or not functioning (25). There is obviously a decrease in the activity of many different DUBs in IHD, so it might be that even the levels of these substitute DUBs are decreased. If that is the case one would expect increased amounts of ubiquitinated proteins and a decrease in the amount of free monoubiquitin in IHD.

USP14 is found in the 19S subunit of the proteasome. It is not clear how important USP14 is for proper functioning of the proteasome, however, it might seems like USP14 is not vital for the performance of the 19S subunit (9,14,15). One interesting possibility is that reduction of USP14 and UCH 37 in IHD might cause reduced levels of proteasome activity. This in turn may trigger apoptosis because cell viability requires intact proteasomal function (69).

Not much is known about the function of USP15. It is the only UBP known to be able to cleave ubiquitin-proline bonds and it might have a growth regulatory role (24). It is hard to tell the consequences of a decrease in the amount of active USP15 and how this will affect the heart function in IHD.

UCH-L3 processes the C-terminus of the ubiquitin-like protein Nedd8. This protein is important for the posttranscriptional modification of proteins and plays a critical role in many cellular processes (26). A decrease in the amount of active UCH-L3 might disrupt these processes, but it is difficult to say what the subsequent consequences would be.

The level of UCH-L1 was unchanged in the IHD samples compared to the controls and the DCM samples. Some data suggests that UCH-L1 might promote apoptosis (15,19,26). It is interesting that a DUB with this function is one of the few DUBs not down regulated or inactivated in IHD. Especially since preliminary data suggests that prolonged exposure of heart tissue to ischemia causes apoptosis.

Analysis of three housekeeping proteins

The fact that tubulin did not turn out to be a good housekeeping protein for these samples were not entirely unexpected. In the literature there are some reports of dysregulation of tubulin, as well as other contractile proteins, in human heart failure (70-73). Five of the six control samples showed no expression of tubulin. Three of these samples ('U', 'F' and 'M') did not show any decrease in active DUBs. Even though these samples did not show any sign of exposure to hypoxia when we ran the western blot for HA, the lack of tubulin expression might suggest that these samples also have experienced a certain degree of ischemia. One of these samples, sample 'M', also showed increased amounts of ubiquitinated proteins when we ran the western blot for Ub. It is clear that the pathology of these samples is complex.

The western blot for actin showed that this protein is even more dysregulated in these samples than tubulin. When we looked in the literature we found that it has been shown that actin is dysregulated in heart failure (70,74-76). All the control samples showed lack of expression of actin. This contributes to the suspicion that all the donor tissues had been exposed to ischemia.

It was surprising that GAPDH was not a good housekeeping protein for these samples. We got this antibody from our collaborators at Harefield who are also working with failed hearts. They had used this antibody in experiments on heart tissues successfully many times and they had never seen

any lack of expression of the protein. A look in the literature did not show any previous observations of dysregulation of GAPDH in heart failure either.

When we had tried three different housekeeping proteins without any luck, we started to think that maybe it was an impossible task to find a protein that was equally expressed in all the samples. We contacted Mike Dunn, which has considerable experience in analyzing protein expression in heart tissues, both healthy and diseased. He told us that he previously had found changes in housekeeper proteins with disease and said it would be difficult to find a protein whose levels reflect total protein levels. He suggested that Coomassie blue staining was the most accurate method to normalize samples.

The Coomassie blue stainings suggested that the loading was fairly equal among the samples, but unfortunately we could not confirm this because of the lack of a good housekeeping protein for the heart tissue samples.

4.4.2 Levels of ubiquitinated proteins

It was not surprising to find an increased amount of ubiquitinated proteins in the DCM samples as it has been shown in the literature that DCM hearts have significantly higher total protein-ubiquitin conjugation compared to control donor heart tissues. Furthermore, it has been shown that some of these proteins are ubiquitinated only in DCM hearts (58).

One would expect that the increased amount of ubiquitinated proteins in DCM would lead to decreased amounts of free ubiquitin because more of the monoubiquitin is attached to proteins. We did not see a decrease in the levels of monoubiquitin in our experiment. Maybe the pool of monoubiquitin is sufficient to accommodate such elevations in polyubiquitin levels.

There were no differences in the levels of ubiquitinated proteins between IHD and healthy donor tissues, and compared to the DCM samples the levels of ubiquitinated proteins were low. An exception was sample ‘M’ which had levels of ubiquitinated proteins similar to the DCM samples even though it belonged to the control group. There are no obvious reasons why there should be increased amounts of ubiquitinated proteins in this sample.

4.4.3 Are the DUBs above the 97 kD mark HAUSP?

Seven heart samples showed lack of HAUSP when we ran the western blot for HAUSP. These comprised 3/3 IHD samples and 4/6 controls. The lack of HAUSP in the IHD samples and two of the controls (lanes 20, 21) was expected since probe-binding did not reveal DUBs at 100 kD. It was surprising however, that the other two control samples (lanes 9, 10) did not show any expression of HAUSP because these samples had bands that we thought corresponded to HAUSP in the probe-binding experiments. It is difficult to explain why western blotting did not show the expression of HAUSP in these two control samples, but the lysates had been stored for a while in the freezer between these two experiments, so it might be that some of the proteins had been broken down.

The combined results from the western blots for HAUSP strongly suggest that the DUB missing at 100 kD in probe-binding experiments was HAUSP. The experiment also confirmed that the absence of HAUSP in the western blot for HA reflects its reduced expression in IHD samples and not just inactivation.

4.4.4 Induction of hypoxic effects in HUVEC

Probe-binding assay

To understand the mechanism underlying DUB loss in ischemia we examined the effects of hypoxia on cultured cells. More than 24 hours of ischemia was necessary to induce down regulation/inactivation of DUBs, but the effect was not as dramatic as for the IHD samples.

It seemed like it was the same DUBs that were affected in this experiment as with the heart tissues. In the lysate from the IHD patients HAUSP and USP14 were entirely absent, while the levels of USP15, UCH37 and UCH-L3 were fairly decreased. The level of UCH-L1 was not changed in the ischemic samples compared to the DCM and controls. In HUVEC exposed to hypoxia the levels of HAUSP and USP14 were obviously decreased, but the bands were not entirely absent. This suggests that more then 96 hours of hypoxia is necessary to create the disappearance of HAUSP and USP14 seen in IHD. In conformity with the IHD samples the level of USP15, UCH37 and UCH-L3 was decreased by 96 hours hypoxia. We suggest, therefore, that changes in DUB

expression in IHD may be initiated by hypoxia. However, it is worth noticing that the level of UCH-L1 was decreased by hypoxia in endothelial cells but was not reduced in IHD. It is not possible to recreate the exact same conditions *in vitro* as found in a healthy heart or a heart suffering from IHD. Some of these differences in environment may be responsible for the differences in the levels of UCH-L1 seen between the HUVEC exposed to hypoxia and the IHD samples.

There was a new DUB appearing in the 96 hours samples, both in the hypoxic sample and in the control. Since it emerged in both samples it is unlikely that the function of this DUB is related to hypoxia. Both samples had been kept in their respective incubators for 96 hours without changing the media. It is therefore possible that this DUB is expressed or activated under conditions with low supply of nutrients and/or high confluence.

Levels of ubiquitinated proteins

More than 8 hours of hypoxia caused a decrease in the level of ubiquitinated proteins in HUVEC. We did not see any changes in the amount of ubiquitinated proteins in the IHD samples. The fact that hypoxic conditions lead to decreased levels of USP14 and UCH37 suggested that if there were any changes in the amount of ubiquitinating proteins there would be an increase in the levels of these proteins because USP14 and UCH37 are involved in the functioning of the proteasome. However, when we ran the western blot for Ub we saw a decrease in the amount of ubiquitinated proteins in the hypoxic samples rather than an increase. It is clear that the biochemistry behind the changes in the levels of ubiquitinated proteins is complex. Moreover, the level of monoubiquitin was decreased in the 24 and 96 hour hypoxia samples and in one of the controls. In theory one would expect the levels of monoubiquitin to be high when the amounts of ubiquitinated proteins are low. It is therefore difficult to explain why the two hypoxic samples show decreased levels of monoubiquitin. The decrease is probably not linked with hypoxia since the 16 hours hypoxia was also associated with low levels of monoubiquitin even though the level of polyubiquitinated proteins in this sample was normal.

We did not see the same result for the two 16 hours of hypoxia samples. In the first experiment (experiment 1) there were a decrease in the level of ubiquitinated proteins, while in the second experiment (experiment 2) we did not see a decrease until 24 hours of hypoxia. In addition, the levels of monoubiquitin did not vary in experiment 1. Small differences in cell confluence, passage and other experimental factors may have been the cause of the different results.

At this stage it is difficult to reconcile the observed changes in UCH37 and USP14 with changes in Ub levels. However, it should be noted that ischemia induces numerous molecular changes in cells. Thus, the relationship between hypoxia, DUBs and Ub may be a complex one.

4.4.5 Hypothesis

It has been shown that inhibition of the proteasome initiates an apoptotic cascade as a result of dysregulation of several proapoptotic proteins (69). Moreover, HAUSP is known to decrease the levels of the tumor suppressor protein p53 through an effect on MDM2. Activation of p53 leads to cell cycle arrest and other alterations that eventually lead to apoptosis (14,22). We have shown that hypoxia leads to decreased levels of USP14, UCH37 and HAUSP. USP14 and UCH37 are involved in proteasome function, and a reduced level of these DUBs could compromise the function of the proteasome, which again could lead to dysregulation of proapoptotic proteins resulting in apoptosis. Decreased level of HAUSP would lead to an increase in the level of p53 and hence increased levels of apoptosis (Figure 4.4.1).

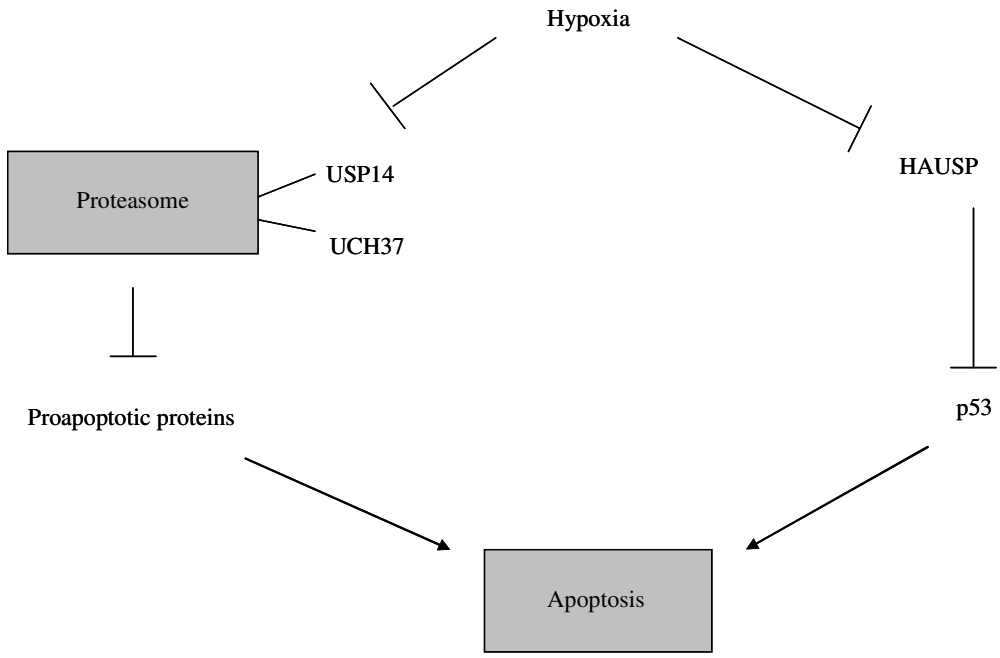

Figure 4.4.1: Hypoxia could promote apoptosis by reducing the expression of USP14, UCH37 and HAUSP.

It has previously been shown that hypoxia induces apoptosis by upregulating several cell death pathways, including the p53 pathway (77). Here we have shown a possible mechanism for the upregulation of the p53 pathway in addition to another mechanism that also could promote apoptosis in cells exposed to hypoxia. We suggest that reduction in USP14, UCH37 and HAUSP levels in response to hypoxia may trigger the apoptotic changes that are characteristic of IHD. Further experiments would be necessary to confirm this theory.

4.5 The effect of hypoxia and IL-1 on the translocation of NF-κB

4.5.1 The effect of hypoxia and IL-1 on the translocation of p65

IL-1 leads to degradation of IκBα and hence nuclear translocation of NF-κB (4). We have previously shown that hypoxia has an effect on the ubiquitin system. With this in mind, we hypothesized that hypoxia might inhibit the translocation of p65 into the nucleus after stimulation of IL-1 by inhibiting the ubiquitination of IκBα and hence its degradation. Our results, however, showed that hypoxia does not inhibit this translocation. Instead hypoxia inhibits the constitutive

translocation of p65 in the absence of an activation signal. We can only speculate in the mechanism behind this inhibition. One possibility is that hypoxia directly inhibits the constitutive translocation of p65, another possibility is that hypoxia activates a mechanism which pumps p65 out of the nucleus after its translocation.

Hypoxia is known to trigger apoptosis in endothelial cells. Therefore it is tempting to speculate that the reduction in constitutive NF-κB, a known survival factor, in endothelial cells exposed to hypoxia may be involved in their susceptibility to apoptosis.

5. CONCLUSIONS

T-cell specific DUBs

- ✓ The novel 50 kD and 60 kD DUBs identified in proliferating CD4+ T-cells, are probably T-cell specific. Neither of the two proteins corresponds to DUB-2.

Ubiquitination and nuclear DUBs in cell cycle regulation

- ✓ There exist at least two nuclear specific DUBs.
- ✓ The activity of one DUB of approximately 40 kD is restricted to the G1 phase of the cell cycle.
- ✓ The levels of ubiquitinated proteins are higher in nuclear lysates than in lysates made from the cytosol.

DUBs in inflammatory bowel disease (IBD)

- ✓ The level of FAM is reduced in intermediate colitis (IC); otherwise the ubiquitin-proteasome system appears to be intact in IBD.

Ubiquitination and deubiquitination in heart failure

- ✓ HAUSP is downregulated in ischemic heart disease (IHD).
- ✓ USP15, UCH37 and UCH-L3 are also down-regulated or inactivated in IHD.
- ✓ The levels of ubiquitinated proteins are elevated in dilated cardiomyopathy (DCM).

Effects of hypoxia on ubiquitination and deubiquitination

- ✓ Exposure of HUVEC to hypoxia results in decreased levels of several DUBs, although the decrease is not as dramatic as in IHD.

- ✓ ≥24 hours of hypoxia decrease the levels of ubiquitinated proteins in HUVEC.

Effects of hypoxia on NF-κB

- ✓ Hypoxia does not inhibit the translocation of p65 into the nucleus after stimulation by IL-1.

- ✓ Hypoxia inhibits the constitutive translocation of p65 into the nucleus.

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APPENDIX

Appendix 1: Printout from the plate reader

C:\REVEL\DUB2NEWT.DAT Printed on 10/11/05 at 2:47:47 PM Page 1 of 3
Plate ID: DUB2newTonje protein concentration

DYNEX REVELATION 3.2

Name :
Address :
Phone :
FAX :

TEST NO.	:	W/L MODE	: SINGLE	DATE	: 5/5/05
TEST NAME	: TONJE	TEST FILTER	: 750 nm	TIME	: 3:53:42 PM
PLATE	: DUB2newTonje	REF. FILTER	: *	OPERATOR	:

Calculation mode : Endpoint

Individual blanks:

A1 = 0.060 B1 = 0.060

DATA MATRIX/TABLE : OD

	1	2	3	4	5	6	7	8	9	10	11	12
A	BLANK	0.045	0.090	0.130	0.157	0.193	0.223	0.259	*****	*****	*****	*****
B	BLANK	0.045	0.091	0.127	0.158	0.190	0.226	0.258	*****	*****	*****	*****
C	0.084	0.131	*****	*****	*****	*****	*****	*****	*****	*****	*****	*****
D	0.085	0.130	*****	*****	*****	*****	*****	*****	*****	*****	*****	*****
E	0.172	0.130	*****	*****	*****	*****	*****	*****	*****	*****	*****	*****
F	0.168	0.126	*****	*****	*****	*****	*****	*****	*****	*****	*****	*****
G	0.159	*****	*****	*****	*****	*****	*****	*****	*****	*****	*****	*****
H	0.154	*****	*****	*****	*****	*****	*****	*****	*****	*****	*****	*****

***** Indicates an unread well or value out of range

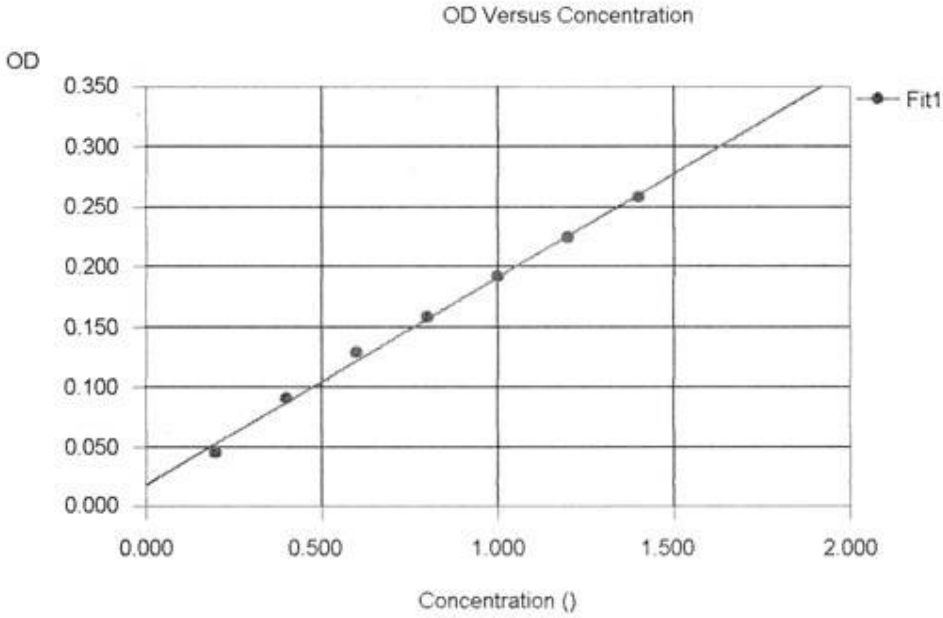
CURVE FITTING

Fit1

Lin/Lin axes scaling
Linear regression with data extrapolation
Y = +0.0178 +0.1735 X

C:\REVEL\DUB2NEWT.DAT Printed on 10/11/05 at 2:47:47 PM Page 2 of 3
Plate ID: DUB2newTonje protein concentration

R-Squared = 0.9965



RESULTS

Sample ID	Location	(OD) Data	(OD) Mean	S.D.	C.V.	Dilution	Conc.
T1	C1 D1	0.084 0.085	0.084	0.001	0.837%	10	3.846
T2	C2 D2	0.131 0.130	0.131	0.001	0.542%	10	6.497
T3	E1 F1	0.172 0.168	0.170	0.003	1.664%	5	4.387
T4	E2 F2	0.130 0.126	0.128	0.003	2.210%	5	3.177
T5	G1 H1	0.159 0.154	0.157	0.004	2.259%	5	3.998

Sample ID	Location	(OD) Data	(OD) Mean	S.D.	C.V.	Dilution	Conc.
S1	A2 B2	0.045 0.045	0.045	0.000	0.000%	1	0.200
S2	A3 B3	0.090 0.091	0.091	0.001	0.781%	1	0.400

C:\REVEL\DUB2NEWT.DAT Printed on 10/11/05 at 2:47:47 PM Page 3 of 3
Plate ID: DUB2newTonje protein concentration

Sample ID	Location	(OD) Data	(OD) Mean	S.D.	C.V.	Dilution	Conc.
S3	A4	0.130	0.129	0.002	1.651%	1	0.600
	B4	0.127					
S4	A5	0.157	0.158	0.001	0.449%	1	0.800
	B5	0.158					
S5	A6	0.193	0.192	0.002	1.108%	1	1.000
	B6	0.190					
S6	A7	0.223	0.225	0.002	0.945%	1	1.200
	B7	0.226					
S7	A8	0.259	0.259	0.001	0.274%	1	1.400
	B8	0.258					

***** Indicates an unread well or value out of range
+++++ Indicates a high out of range result
----- Indicates a low out of range result
# Indicates extrapolated data

DYNEX TECHNOLOGIES